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Conséquences de la domestication du riz sur son principal agent pathogène fongique, *Magnaporthe oryzae*: structure des populations, dispersion, et évolution du régime de reproduction.

Jury

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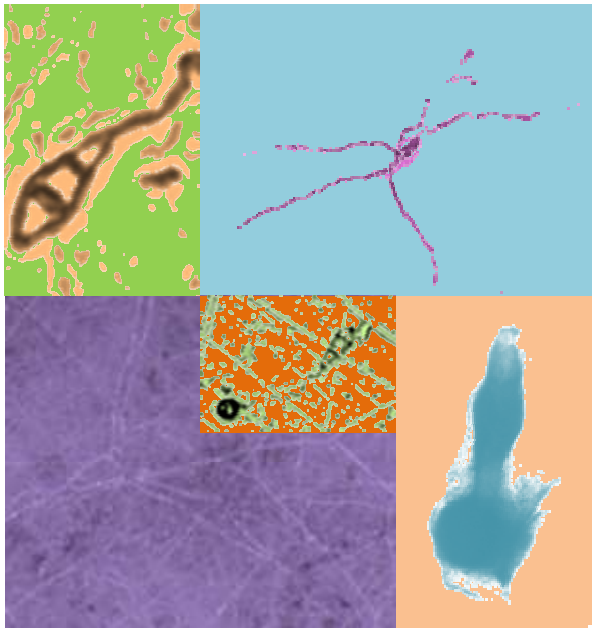
INTRODUCTION



La domestication a eu des conséquences évolutives importantes sur les agents pathogènes associés aux plantes cultivées. Cette thèse a pour but d'améliorer la compréhension de certains des mécanismes évolutifs ayant un lien direct ou indirect avec la domestication, qui ont été impliqués dans l'évolution de ces agents pathogènes.

I. Le problème des agents pathogènes d'espèces domestiquées : un défi pour l'agriculture.

1. Importance des agents pathogènes menaçant les espèces domestiquées.

L'évolution d'un grand nombre d'espèces a été influencée par l'Homme afin de les exploiter plus facilement. Ces espèces qui ont été modifiées par rapport à leur(s) ancêtres sauvage(s), sont élevées/cultivées dans des systèmes anthropisés et leur alimentation ainsi que leur reproduction sont contrôlées par les humains. C'est la définition que Diamond (2002) a donné de la domestication. Les processus de domestication sont étalés dans le temps, puisque la sélection exercée pour modifier plusieurs traits chez une espèce nécessite un grand nombre de générations. La domestication est restreinte géographiquement pour un grand nombre d'espèces pour lesquelles elle a eu lieu dans une aire unique. C'est le cas par exemple du maïs qui a été domestiqué depuis la téosinte au Mexique (Hastorf 2009).

L'Homme a dû faire face au problème des parasites dès l'apparition de l'agriculture pendant la révolution Néolithique il y a 12000 à 2000 ans (Balter 2007 ; Stukenbrock & McDonald 2008). Ces parasites incluent des insectes herbivores, occasionnant des dégâts physiques sur les plantes, et des agents pathogènes, microorganismes affectant la capacité de survie, de croissance, de reproduction, de compétition et de défense contre d'autres agents pathogènes ou herbivores (Jarosz & Davelos 1995). L'émergence et la dispersion de nouveaux génotypes d'agents pathogènes ont été favorisées par l'intensification de l'agriculture, l'homogénéisation des espèces et variétés cultivées, ainsi que par l'augmentation des échanges de plantes (Anderson *et al.* 2004). Il existe de nombreux exemples d'épidémies d'agents pathogènes détruisant des récoltes entières et provoquant des famines. Par exemple, l'Irlande a connue une famine entraînant la mort de plus d'un million de personnes au XIXe siècle, provoquée par un champignon *Phytophtora infestans* qui a dévasté les cultures de pomme de terre (Stukenbrock & McDonald 2008). Malgré les efforts

dans la lutte contre les bioagresseurs, les agents pathogènes de plantes cultivées constituent toujours une menace pour l'alimentation humaine dans différentes région du monde, en particulier dans les pays en développement (Strange & Scott 2005 ; Pennisi 2010). Les maladies de plantes sont d'autant plus problématiques qu'une grande partie affecte les cultures composant l'alimentation de base, comme le riz, le blé, le maïs ou la pomme de terre (Anderson *et al.* 2004). Plus de 40% de la production agricole mondiale de ces trente dernières années a en effet été réservée à ces espèces.

2. Co-évolution entre hôte et agent pathogène dans un milieu cultivé.

L'environnement d'un agent pathogène est représenté en partie par son hôte. L'hôte, quant à lui, évolue en interaction avec le parasite et est soumis par ailleurs à des pressions de sélection plus ou moins fortes de son environnement. Ainsi, hôte et parasite s'imposent mutuellement des pressions de sélection antagonistes qui font qu'ils évoluent l'un en réponse de l'autre. Van Valen (1973) a détourné une métaphore tirée du conte d'Alice au pays des merveilles pour illustrer les interactions entre espèces. Alice qui court après la Reine Rouge, remarque que ni l'une ni l'autre ne change de place malgré l'effort. La Reine Rouge lui répond alors que de rester à la même place nécessite de courir aussi vite que possible. L'hypothèse formulée par Van Valen, appelée hypothèse de la Reine Rouge, suggère une course évolutive constante entre les espèces qui sont en interaction. Ainsi un hôte peut développer des résistances à un agent pathogène, et l'agent pathogène peut contourner ces résistances. Par la domestication, l'Homme a influencé ces pressions de sélection subies par l'hôte en modifiant l'environnement de l'hôte et en lui imposant directement une sélection sur certains traits. Cette sélection a affecté non seulement l'évolution des hôtes mêmes mais aussi l'évolution des espèces avec lesquelles ils interagissent, dont les agents pathogènes. Dans un système cultivé, l'évolution de l'hôte est entièrement contrôlée par l'Homme, alors que celle de l'agent pathogène se fait de manière libre. Ainsi, quand un agent pathogène contourne un génotype d'hôte portant un système de résistance donné, il peut envahir rapidement la population d'hôtes puisque tous les individus portent la même résistance. En revanche, la plante ne co-évolue pas librement en réponse à l'agent pathogène mais son évolution est contrôlée par l'Homme via la création variétale. Les changements évolutifs des agents pathogènes en interaction avec leur plante hôte sont donc considérés comme plus rapides dans un écosystème cultivé que dans un écosystème naturel (Stukenbrock & McDonald 2008 ;

McDonald & Linde 2002 ; Stahl & Bishop 2000 ; Zhan *et al.* 2002). En effet, certains agents pathogènes sont capables de contourner les résistances d'une variété en moins d'un an. Ainsi, les plantes cultivées sont largement représentées dans la littérature comme des modèles pour l'étude de l'évolution d'agents pathogènes (Hancock 2005).

La plupart des études sur la domestication s'intéressent aux conséquences de la sélection artificielle sur la variabilité génétique des organismes domestiqués. En revanche, beaucoup de connaissances restent à acquérir sur les conséquences de la domestication sur l'évolution des cortèges parasitaires associés aux organismes domestiqués (Buckler *et al.* 2001), même si les études se développent (Stukenbrock & McDonald 2008). La domestication a modifié l'environnement des agents pathogènes : la disponibilité en hôtes a été augmentée puisque les populations d'hôtes ont une taille plus importante et sont génétiquement homogènes. Ainsi, les mécanismes adaptatifs des agents pathogènes sont susceptibles d'avoir été modifiés dans un tel environnement. Il est utile de comprendre quelles conséquences la domestication a eu sur les agents pathogènes pour mieux gérer les maladies qu'ils causent et anticiper leur évolution. Parmi les agents pathogènes de plantes, les champignons sont responsables de presque un tiers des maladies émergentes observées (Anderson *et al.* 2004 ; Giraud *et al*. 2010), ce qui en fait des modèles particulièrement intéressants.

Cette première partie a pour but de proposer une synthèse sur l'influence de la domestication et de l'intensification de l'agriculture sur les espèces pathogènes associées aux plantes cultivées, en particulier sur les champignons phytopathogènes.

II. Evolution des plantes avec la domestication et l'intensification de l'agriculture.

Pour déterminer les conséquences évolutives de la domestication sur les agents pathogènes, il est nécessaire de rappeler les modifications qu'a entrainées la domestication sur les plantes elles-mêmes (Figure 1.1). Certains de ces changements évolutifs découlent directement du processus de domestication, c'est-à-dire de sélection de génotypes. D'autres changements évolutifs découlent de processus graduels directement liés à la domestication : l'intensification de l'agriculture et l'augmentation des échanges commerciaux. Ces processus se sont accélérés avec la révolution industrielle au XIXe siècle et après la Seconde Guerre Mondiale.

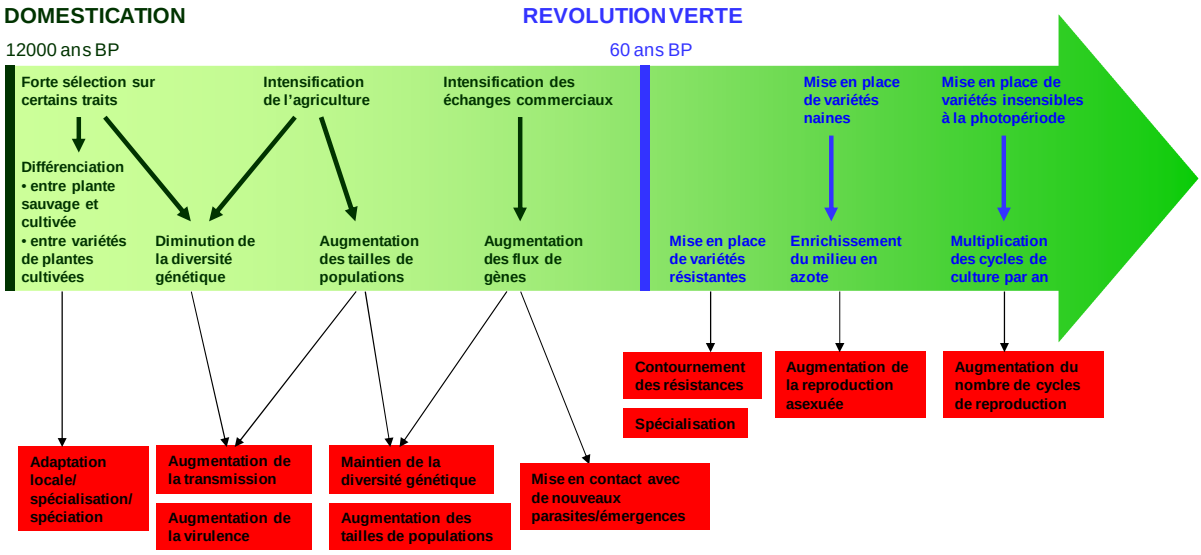


Figure 1.1. Evolution des hôtes et parasites dans un milieu cultivé.
Effets de la domestication, de l'intensification de l'agriculture, des échanges commerciaux, et de la révolution verte sur l'évolution des plantes cultivées (vert) et les conséquences sur les agents pathogènes associés (rouge).

1. Changements évolutifs liés à la domestication

Chez de nombreuses plantes cultivées, y compris les céréales, la domestication a entraîné des modifications phénotypiques similaires. C'est ce qui a été appelé le syndrome de domestication (Li *et al.* 2006). Par exemple, des traits liés à la période de floraison, à la taille des graines et à la taille des plantes ont été sélectionnés chez les Poacées (Buckler *et al.* 2001) et d'autres plantes cultivées. Chez les céréales, la qualité des grains —leurs propriétés gustatives, l'absence d'aristation, ou encore la couleur— a été sélectionnée, alors que l'égrenage a été contre-sélectionné (Figure 1.2). La domestication aurait impliqué la sélection de mutations en relation avec ces traits sur seulement quelques loci mais ayant des effets potentiellement importants. Ainsi, chez des espèces différentes de Poacées qui ont été domestiquées indépendamment, les mêmes loci ont été sélectionnés (Buckler *et al.* 2001). Par exemple, la sélection pour la taille des graines, la dispersion des graines et la photopériode lors de la domestication impliquent des loci de traits quantitatifs (QTL) qui se retrouvent dans des régions homologues chez le maïs, le riz et le sorgho (Paterson *et al.* 1995).

Cette sélection très forte s'est accompagné de forts goulots d'étranglement qui ont diminué significativement la diversité génétique de l'espèce domestiquée (Londo *et al.* 2006). Par exemple, chez les Poacées, la plupart des espèces domestiquées présentent environ les deux tiers de la diversité nucléotidique observée chez leur ancêtre sauvage, comme par exemple le maïs, le sorgho et l'avoine (Buckler *et al.* 2001). Certaines espèces domestiquées présentent une diversité encore plus réduite, comme par exemple le blé qui ne présente que 30% de la variabilité génétique de ses ancêtres sauvages. Quant au riz cultivé, *Oryza sativa*, 71% de la diversité génétique a été conservée par rapport à l'ancêtre sauvage *O. rufipogon* (Buckler *et al.* 2001).

2. Intensification de l'agriculture et des échanges commerciaux

A la suite de la domestication, la culture de plantes, en particulier de céréales, s'est intensifiée pour subvenir aux besoins des populations humaines. Les populations de plantes cultivées se sont trouvées augmentées en taille et en homogénéité génétique. L'intensification des échanges commerciaux a aussi entrainé une augmentation des flux de gènes de plantes

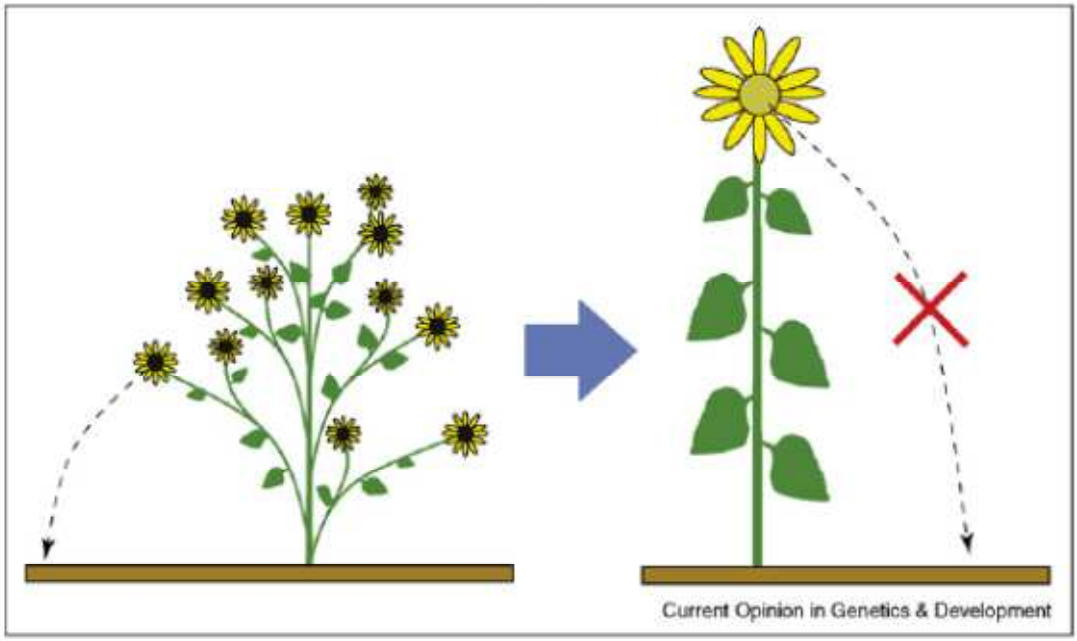


Figure 1.2. Effets de la domestication sur l'évolution du tournesol.
(d'après Burke *et al.*, 2007)
Les traits sélectionnés chez la plupart des plantes cultivées sont : l'augmentation de la tailles des fruits ou des graines, augmentation de la croissance et de la dominance apicale (peut de ramifications), suppression de la dispersion naturelle par les graines, perte de la dormance des graines et perte de l'auto-incompatibilité.

cultivées entre différentes régions, ce qui a participé à maintenir de la diversité génétique. Les échanges commerciaux ont aussi nécessité d'adapter les plantes cultivées à de nouvelles conditions environnementales, en particulier climatiques, différentes de l'aire d'origine de leur ancêtre sauvage.

3. La Révolution Verte

Cependant, ces soixante dernières années, des milliers de variétés traditionnelles ont été remplacées par un nombre réduit de variétés modifiées pour augmenter le rendement, en particulier pour le riz et le blé. Cette homogénéisation soudaine des cultivars à l'échelle mondiale a été appelée « Révolution Verte » et la diversité génétique de ces cultures s'en est trouvée fortement réduite (Khush 2001). Entre autres modifications, la mise en place de variétés naines a permis l'augmentation de l'utilisation des engrais azotés, augmentant ainsi le rendement (Figures 1.3 et 1.4). La réduction du temps du cycle de production et l'insensibilité à la photopériode ont permis d'augmenter le nombre de cycles de culture par an. Enfin, les variétés ont été améliorées pour la résistance aux maladies et ravageurs. Ces changements ont entrainé une modification des pratiques culturales allant dans le sens d'une intensification dans l'espace, mais aussi dans le temps.

III. Conséquences de la domestication et de l'intensification de l'agriculture sur l'évolution des agents pathogènes.

1. Apparition des agents pathogènes sur les plantes domestiquées.

a. Mécanismes d'émergence des agents pathogènes sur les plantes domestiquées.

Les mécanismes d'émergence des agents pathogènes sont variables, même si l'on peut retrouver des similarités entre les agents pathogènes de plantes domestiquées et les agents pathogènes d'animaux domestiqués (Encadré). L'émergence d'un agent pathogène sur une nouvelle espèce cultivée peut avoir lieu au moment même de la domestication de la plante par

Figure 1.3. Morphologie de différents cultivars de riz avant et après la révolution verte. (d'après Khush, 2001)
A gauche : plante de grande taille précédant la révolution verte
A centre : plante améliorée avec un rendement élevé et un tallage important
A droite : plante améliorée avec peu de tallage et robuste (« Super Rice »).

Figure 1.4. Effet de l'ajout d'engrais azotés sur une variété traditionnelle (à gauche) et sur une variété naine mise en place avec la révolution verte (à droite) de riz cultivé *Oryza sativa.* L'azote des engrais visant à améliorer le rendement a pour effet secondaire d'augmenter la croissance de la tige. Dans le cas d'une variété traditionnelle non naine, la plante s'affaisse.
Photo : D. Saleh.

Encadré : Qu'en est-il des agents pathogènes d'animaux domestiqués ?

Certains mécanismes évolutifs liés à la domestication chez les agents pathogènes de plantes cultivées sont aussi observés chez les agents pathogènes d'animaux domestiqués.

Par exemple, une conséquence souvent observée de la domestication d'espèces animales et de leur introduction dans de nouvelles aires est la diminution de la diversité génétique chez leurs agents pathogènes. C'est le cas, par exemple, de *Trichinella spiralis*, un nématode pathogène du porc domestiqué et transmissible à l'Homme. Les isolats de *T. spiralis* provenant d'Asie présentent une diversité génétique significativement plus importante que les isolats provenant d'élevages d'Europe et du continent américain, ces derniers présentant des allèles fixés pour un grand nombre de marqueurs microsatellites et mitochondriaux (Rosenthal, 2008). L'Asie étant la première zone de domestication du porc, Rosenthal (2008) suggère une origine unique de *T. spiralis* sur porc domestiqué et une dispersion depuis le centre d'origine vers le reste du monde, s'accompagnant d'une forte réduction de la diversité génétique. Les autres exemples d'agents pathogènes d'animaux dont la distribution et la diversité génétiques ont été influencées par l'Homme sont nombreux (*Sarcocystis cruzi* sur bétail, *Toxoplasma gondii* sur félins, *Francisella* sur poisson…) mais ne seront pas détaillés ici, puisque nous nous focaliserons sur les agents pathogènes de plantes.

Par ailleurs, les conditions d'élevage ont été aussi des facteurs de propagation d'agents pathogènes puisque la densité importante d'hôtes a augmenté la transmission des agents pathogènes (Lebarbenchon *et al.*, 2008).

La domestication d'espèces animales est susceptible d'avoir augmenté le nombre de sauts d'hôte de leurs agents pathogènes. Ce mécanisme est beaucoup étudié puisqu'il a été suggéré que la transmission des agents pathogènes à partir d'espèces animales vers l'Homme avait augmentée avec la domestication (Diamond, 2002, Pearce-Duvet, 2006 ; Rosenthal, 2008). Par conséquent, les sauts d'hôte d'agents pathogènes sur l'Homme sont plus fréquents depuis les espèces domestiquées que depuis les espèces sauvages d'origine, ce qui peut s'expliquer par l'augmentation de la fréquence des contacts avec les pratiques liées à la domestication (tailles de population de bétail importantes). C'est par exemple le cas de la rougeole et de la tuberculose qui proviennent d'agents pathogènes du bétail et plus récemment des grippes provenant de la volaille et des porcs (Diamond, 2002). L'augmentation de la densité de population humaine a eu pour conséquences, d'un point de vue épidémiologique, le maintien de populations d'agents pathogènes stables, l'augmentation de la transmission d'agents pathogènes depuis les animaux domestiqués, et l'exposition à de nouveaux agents pathogènes due à l'expansion géographique humaine (Zaffarano *et al.*, 2008 ; Diamond, 2002).

host-tracking. Il s'agit de la coévolution entre hôte et agent pathogène pendant la domestication de l'hôte, ou encore la domestication de l'agent pathogène avec l'hôte (Stukenbrock & McDonald 2008). L'ancêtre de l'agent pathogène sur plante domestiquée est donc l'agent pathogène qui était présent sur la plante sauvage. L'hôte ayant subi une diminution de la diversité génétique au cours du processus de domestication, l'agent pathogène sélectionné sur cet hôte est susceptible de subir aussi un goulot d'étranglement (Hoberg & Brooks 2008 ; Rosenthal 2008). C'est en effet le cas chez le champignon pathogène du blé *Mycosphaerella graminicola* qui présente des populations pathogènes sur une gamme étendue de Poacées sauvages, alors que l'agent pathogène domestiqué est spécialisé sur le blé (Stukenbrock *et al.* 2010). *P. infestans* a aussi co-évolué avec son hôte, la pomme de terre, au cours de la domestication de celle-ci (Gomez-Alpizar *et al.* 2007).

Un deuxième mode d'émergence d'un agent pathogène sur une plante cultivée est le saut d'hôte depuis une espèce génétiquement proche du nouvel hôte (*host shift*), ou alors depuis un hôte génétiquement éloigné (*host jump*). Dans ce cas, le centre d'origine de l'hôte et celui de l'agent pathogène peuvent être différents (Stukenbrock & McDonald 2008). L'agent pathogène est mis en contact avec la plante après introduction de l'un ou l'autre dans une nouvelle aire. L'agent pathogène occupe alors une nouvelle niche écologique constituée par l'hôte (Facon *et al.* 2006). Par exemple, il y a eu un saut d'hôte du champignon phytopathogène *Rynchorium secalis* depuis le seigle sur l'orge lors de l'introduction de l'orge en Europe (Brunner *et al.* 2007). Ainsi, le centre de domestication de l'orge (Moyen Orient) ne correspond pas au centre d'origine de l'agent pathogène sur la plante. Giraud *et al.* (2010) suggèrent que le saut d'hôte soit le mécanisme le plus courant dans l'émergence de nouveaux agents pathogènes.

Enfin, un agent pathogène peut émerger sur un nouvel hôte grâce à un transfert de matériel génétique. La quantité de matériel échangé est plus ou moins importante (gène, chromosome, génome). Le transfert horizontal de gènes (*HGT*) permet à un agent pathogène non virulent de devenir virulent sur un nouvel hôte en acquérant des gènes de pathogénicité d'une espèce pathogène. Par exemple, un gène de virulence sur le blé cultivé (*ToxA*) a été transféré depuis le champignon *Phaeosphaeria nodorum* sur *Pyrenophora tritici-repentis*. Le transfert horizontal de mini-chromosomes entre des lignées de *Fusarium oxysporum*, a converti des lignées non pathogènes en lignées pathogènes de la tomate (Ma *et al.* 2010). Le champignon *Aspergillus clavatus* a acquis le gène d'avirulence *ACEI* par HGT à partir d'un donneur proche de *Magnaporthe oryzae* (Khaldi *et al.* 2008). Quand le transfert de matériel

génétique porte sur une portion de génome, on parle d'introgression d'une espèce vers une autre. Enfin, l'hybridation entre différents agents pathogènes permet un échange de gènes entre eux, comme le transfert horizontal de gènes, mais à l'échelle du génome. L'évolution rapide d'agents pathogènes par hybridation est encore relativement méconnue (Arnold 2004). Il existe de rares exemples, comme celui de l'hybridation des deux sous-espèces de champignons pathogènes de l'orme *Ophiostoma novo-ulmi* et *O. novo-americana* (Konrad *et al.* 2002) ou celui de l'hybridation des sous espèces *uniformis* et *multiformis* de *Phytophthora alni* pour donner la sous espèce *alni*.

Dans le cas d'une émergence par *host-tracking*, le centre d'origine de l'agent pathogène correspond au centre de domestication de l'hôte. En ce qui concerne les mécanismes de saut d'hôte et de transfert de matériel génétique, le centre d'origine de l'agent pathogène peut être en dehors de l'aire de domestication de l'hôte. Déterminer le centre d'origine d'un agent pathogène est donc un élément clé pour comprendre les conditions de son émergence sur une plante domestiquée.

b. Adaptation locale, spécialisation et spéciation.

Lors de la domestication d'une plante, il peut y avoir une diminution de la diversité génétique chez un agent pathogène suivant la diminution de la diversité génétique de la plante hôte. Par exemple, Wichmann *et al.* (2005) ont mis en évidence une diminution de la variabilité génétique chez la bactérie X*anthomonas axonopodis* pv. *vesicatoria* pathogène du poivron et de la tomate, traduisant un goulot d'étranglement concordant avec la domestication et l'évolution des pratiques agricoles.

Lorsqu'un agent pathogène est domestiqué avec son hôte, les flux de gènes entre les populations d'agents pathogènes évoluant sur l'hôte sauvage et celles évoluant sur l'hôte domestiqué sont progressivement réduits, ce qui peut entrainer la spécialisation, voire la spéciation de l'agent pathogène sur l'hôte cultivé. A l'inverse, il existe des exemples d'agents pathogènes pouvant infecter l'hôte sauvage et l'hôte cultivé, comme *Ustilago maydis*, champignon pathogène du maïs et de son ancêtre sauvage, la téosinte (Stukenbrock & McDonald 2008).

De plus, sur l'hôte domestiqué, les flux de gènes de l'agent pathogène peuvent être maintenus entre différents groupes génétiques de l'hôte, ou alors restreints, voire limités, à un

groupe génétique. Si les flux de gènes d'un agent pathogène sont restreints à une espèce ou une variété d'hôte, il y a spécialisation. Si cette spécialisation a lieu à l'échelle d'une population d'hôtes, il y a adaptation locale de l'agent pathogène à cette population. Enfin, si les flux de gènes de l'agent pathogène sont exclusivement limités à une espèce ou une variété, il y a spéciation de l'agent pathogène sur un hôte (Sicard *et al.* 2007).

L'adaptation locale est favorisée par les pressions de sélection appliquées par l'hôte sur l'agent pathogène et inversement par l'agent pathogène sur l'hôte (Greischar & Koskella 2007). Ainsi, si un génotype d'hôte est plus représenté que les autres dans une population d'hôtes, un temps de génération plus court et une taille de population plus importante chez l'agent pathogène lui confèrent un avantage pour s'adapter localement (Ebert, 1994 ; Hamilton *et al.* 1990; Gandon & Michalakis 2002 ; Greischar & Koskella 2007). Si l'agent pathogène est localement adapté à un génotype d'hôte, la mise en place d'une nouvelle variété d'hôte par l'Homme peut réduire très rapidement la population d'agents pathogènes. L'adaptation locale est aussi favorisée par des flux migratoires entre populations. En effet, l'introduction de nouveaux allèles dans une population d'agents pathogènes peut lui permettre de contourner plus rapidement la résistance de l'hôte (Gandon & Michalakis 2002 ; Greischar & Koskella 2007 ; Criscione 2008). L'intensification des échanges commerciaux de plantes a donc été susceptible, par l'augmentation des flux de gènes d'agents pathogènes entre différents champs et la dispersion de matériel infecté, de favoriser l'adaptation locale.

Si la spécialisation est quantitative, l'agent pathogène est plus performant sur un hôte particulier mais reste capable d'infecter d'autres génotypes. Par exemple, Sicard *et al.* (2007) ont mis en évidence une spécialisation de certaines populations du champignon *Colletotrichum lindemuthianum* à deux espèces hôtes de haricots *Phaseolus vulgaris* et *P. coccineus*. Il existe des mécanismes quantitatifs qui affectent l'agressivité de champignons pathogènes de plantes, c'est-à-dire la capacité à envahir les cellules et à produire des spores.

Si la spécialisation est qualitative, l'agent pathogène n'est plus capable d'infecter qu'un seul génotype d'hôte. Une spécialisation qualitative peut avoir pour conséquence une spéciation. Par exemple, il y a eu spéciation du champignon *Rynchosporium secalis* sur l'orge, le seigle et *Agropyron sp.* (Zaffarano *et al.* 2008). D'après Giraud *et al.* (2010), il existe un certains nombre de traits présents chez les champignons pathogènes de plantes qui peuvent permettre une spéciation écologique à un nouvel hôte de manière rapide parmi lesquels une sélection disruptive forte imposée par l'hôte, un taux de sporulation important, une reproduction sexuée intra hôte, un faible nombre de gènes contrôlant la spécificité de

l'interaction hôte-pathogène et une reproduction asexuée importante ponctuée de quelques évènements de reproduction sexuée. Chez les plantes infectées par des champignons pathogènes, il existe aussi des mécanismes de résistance qualitatifs généralement contrôlés par des relations gène pour gène : un gène de résistance chez la plante interagit avec un gène d'avirulence chez le champignon.

L'homogénéisation des génotypes de plantes entrainée par la domestication, et surtout l'intensification de l'agriculture, a favorisé la spécialisation ou même la spéciation d'agents pathogènes sur une espèce ou une variété d'hôte (Stukenbrock & McDonald 2008). C'est pourquoi la divergence entre différentes espèces d'agent pathogènes appartenant à un même genre est souvent considérée comme postérieure à la domestication et à la différenciation des espèces hôtes. C'est le cas des espèces du genre *Puccinia* qui provoquent la rouille sur les feuilles de blé, des espèces du genre *Blumeria* qui provoquent l'oïdium sur blé et des espèces du genre *Ustilago* et *Sporisorium* qui provoquent le charbon sur un certains nombre de céréales (Muncaksi *et al.* 2007). Il existe aussi des cas d'agents pathogènes ayant divergé avant la domestication de leurs hôtes. Ainsi, Muncaksi *et al.* (2007) ont mis en évidence une divergence des espèces *U. maydis*, *U. scitaminea*, *S. reilianum* et *S. sorghi* antérieure à la domestication de leurs hôtes respectifs : le maïs pour *U. maydis*, la canne à sucre pour *U. scitaminea* et *S. reilianum* et le sorgho pour *S. sorghi*.

2. Conséquences de l'intensification de l'agriculture

L'intensification de l'agriculture a modifié le milieu dans lequel évoluent les agents pathogènes. Une première conséquence importante de cette intensification est l'augmentation des populations d'hôtes (en nombre et en surface), génétiquement identiques, disponibles pour les agents pathogènes. Ceci a été susceptible de limiter la dérive génétique et donc de maintenir un niveau de diversité important chez les agents pathogènes (Caillaud *et al.* 2006). La disponibilité en hôte est aussi susceptible d'avoir influencé le mode de reproduction des agents pathogènes. De plus, les pratiques culturales, comme l'utilisation de pesticides ou de variétés résistantes, ont eu pour conséquences la sélection et l'augmentation en fréquence des génotypes des agents pathogènes adaptés aux conditions de culture (McDonald & Linde 2002).

a. Evolution des tailles de populations d'agents pathogènes.

La structure et la dynamique des populations d'agents pathogènes ont été modifiées avec le développement des systèmes agricoles (Couch *et al.* 2005 ; Stukenbrock *et al.* 2007 ; Munkacsi *et al.* 2008 ; Zaffarano *et al.* 2008 ; Gladieux *et al.* 2010). Ces modifications concernent non seulement les agents pathogènes mais aussi les adventices de plantes cultivées. Par exemple, les tailles de populations de *Agropyron sp.*, adventice de plusieurs céréales, a augementé avec l'intensification de l'agriculture (Zaffarano *et al.* 2008). Chez les agents pathogènes de plantes, la même tendance est observée. Par exemple, chez *M. graminicola*, champignon pathogène du blé, l'augmentation des tailles de populations de blé cultivé a entrainé l'augmentation des tailles de populations de l'agent pathogène (Stukenbrock *et al.* 2007). Il en est de même pour *R. secalis* sur le seigle et l'orge. L'augmentation des tailles de populations d'agents pathogènes a des conséquences importantes sur leur diversité génétique. En effet, en limitant la dérive génétique elle permet de maintenir un niveau de diversité important (Stukenbrock & Mc Donald 2008 ; Gladieux *et al.* 2010). D'autre part, chez les plantes annuelles, la mise en place de cycles de culture tout au long de l'année permet aux agents pathogènes spécialisés de persister plus longtemps puisqu'ils ne subissent pas de goulot d'étranglement lié à l'interruption de la culture (Khush 2001).

b. Evolution du cycle de vie.

Une autre conséquence évolutive de l'intensification de l'agriculture sur leurs agents pathogènes est l'augmentation du nombre de cycles de reproduction (sexuée et asexuée) par la multiplication des cycles de culture pendant l'année. La taille efficace des populations d'agents pathogènes de plantes cultivées s'en trouve donc également augmentée.

Chez les espèces qui peuvent se reproduire à la fois de manière sexuée et asexuée comme la plupart des champignons phytopathogènes, une conséquence possible de la domestication est la sélection pour plus de reproduction asexuée en comparaison de la reproduction sexuée. En effet, la reproduction asexuée est favorisée dans un milieu favorable au développement de l'agent pathogène comme c'est généralement le cas dans les systèmes

cultivés. Dans un milieu moins favorable, la reproduction sexuée, dont le temps de génération est généralement plus long, va être favorisée (Stukenbrock & McDonald 2008).

La reproduction sexuée jouant un rôle important dans l'adaptabilité des agents pathogènes à leur hôte, il est primordial de déterminer le mode de reproduction d'un agent pathogène dans les différentes zones de culture de son hôte.

c. Evolution de la transmission et de la virulence.

L'intensification de l'agriculture a eu des effets très forts sur la virulence et la capacité de transmission d'agents pathogènes associés aux plantes. Il existe un compromis entre virulence et transmission chez un grand nombre d'espèces pathogènes. Ainsi, un agent pathogène trop virulent, c'est-à-dire qui exploite trop rapidement les ressources de son hôte, risque de le tuer avant d'être transmis sur un nouvel hôte. Or, le taux de reproduction d'un agent pathogène dans son hôte est un facteur important de la virulence et la capacité de transmission de cet hôte augmente avec son taux de reproduction (Figure 1.5, Ebert & Bull 2003). Dans une revue, Froissart *et al.* (2010) montrent qu'une corrélation positive entre accumulation virale et transmission est retrouvée dans de nombreux systèmes virus-hôtes.

En ce qui concerne les agents pathogènes de plantes cultivées, ce compromis est modifié. Les fortes densités des populations hôtes et l'homogénéité des génotypes permettent une transmission plus efficace de l'agent pathogène d'une plante à l'autre par rapport à un écosystème naturel (Stukenbrock & McDonald 2008). Elles modifient ainsi le compromis entre virulence et transmission puisque même les souches très virulentes peuvent envahir ce milieu dense et homogène (Thrall & Burdon 1999).

3. Conséquences des échanges commerciaux.

Les populations d'agents pathogènes de plantes cultivées sont stabilisées par des densités d'hôte plus importantes, une homogénéité de l'hôte et une dispersion augmentée par les échanges permettant une transmission à une échelle importante (Zaffarano *et al.* 2008).
En effet, des migrations récurrentes participent à maintenir la variabilité

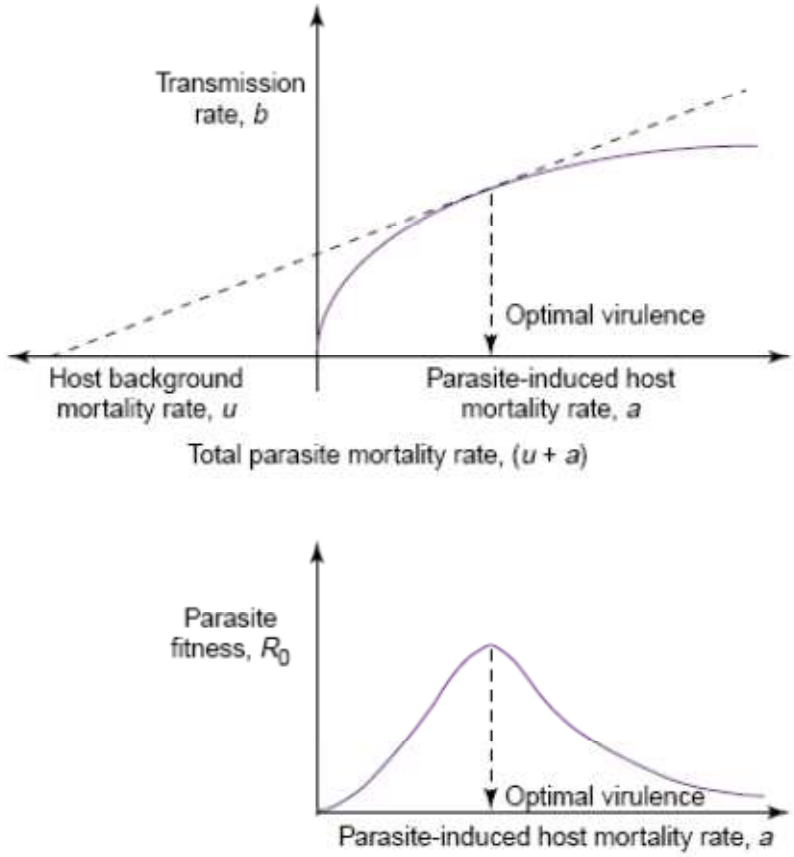


Figure 1.5. Compromis entre virulence et transmission.
(d'après Ebert & Bull, 2003)
La relation entre virulence et transmission est une fonction croissante dont la pente diminue. La mort rapide de l'hôte affectant la transmission du parasite, il existe une valeur optimale de virulence pour la transmission du parasite.

génétique (Salvaudon *et al*., 2008). La multiplication des échanges commerciaux a entrainé, par le transport de matériel végétal infecté, la dispersion des agents pathogènes vers de nouvelles aires (Brown & Hovmoller 2002). Les processus impliqués dans l'établissement d'un agent pathogène dans une nouvelle aire peuvent être assimilés à ceux décrits pour les espèces invasives (Dlugosh & Parker 2008a ; Keller & Taylor 2008 ; Whitney & Gabler 2008). Les agents pathogènes se sont adaptés à de nouvelles conditions. Certains agents pathogènes ont subi des goulots d'étranglement alors que d'autres se sont diversifiés dans des aires d'introduction. Le régime de reproduction est susceptible d'avoir été modifié dans les aires d'introduction. L'histoire évolutive de certains agents pathogènes est donc complexe et sa compréhension nécessite de déterminer les centres d'origine, de diversité et de dispersion.

Nous avons vu que déterminer le centre d'origine, de diversité et de dispersion d'un agent pathogène est important pour comprendre les mécanismes évolutifs ayant façonné notamment la diversité génétique et la distribution géographique observée. Par ailleurs, nous avons vu que l'évolution du mode de reproduction était influencée par la domestication et les pratiques agricoles. Ces deux points sont essentiels à la compréhension de l'évolution des agents pathogènes de plantes et représentent les deux axes de cette thèse. Nous avons choisi comme modèle *Magnaporthe oryzae*, champignon pathogène du riz domestiqué.

IV. Présentation du pathosystème.

1. Présentation de *Magnaporthe oryzae*.

M. oryzae est le champignon ascomycète responsable de la principale maladie fongique sur le riz, la pyriculariose. *M. oryzae* forme un complexe d'espèces avec, entre autres, une espèce non nommée pathogène de *Pennisetum spp* et *M. grisea* pathogène de *Digitaria spp* qui peut être distinguée sur la base du polymorphisme de gènes nucléaires, de la spécificité d'hôte et par des tests de fertilité (Couch et Kohn 2002 ; Zellerhoff *et al.* 2006). *M. oryzae* comprend différents groupes génétiques, pathogènes du riz et de différentes espèces de Poacées dont le blé, le maïs et les sétaires. La forme imparfaite de *M. oryzae*, ou forme asexuée, est appelée *Pyricularia oryzae* (Ebbole 2007).

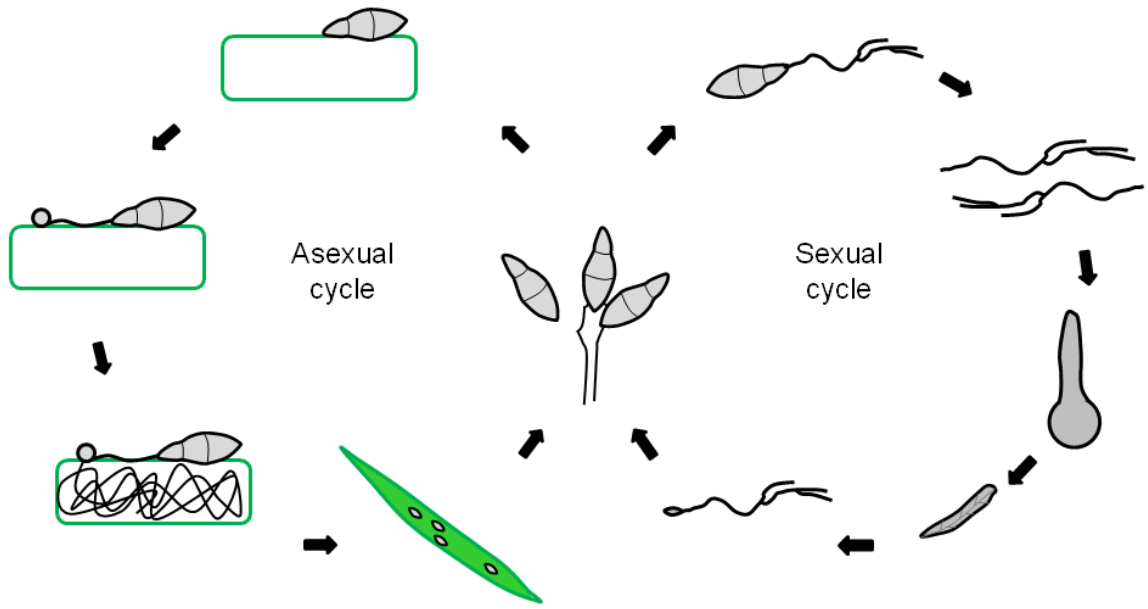


Figure 1.6. Cycle de vie de *Magnaporthe oryzae*.

M. oryzae est un champignon hémibiotrophe qui se développe sur les feuilles, les tiges, les inflorescences et les grains de riz. L'infection des racines a aussi été observée (Sesma & Osbourn 2004 ; Guerber & Teebest 2006 ; Marcel *et al.* 2010). Le cycle infectieux qui est asexué (Talbot 2003) a été largement décrit (Figure 1.6). A partir du mycélium, le champignon forme des conidiophores portant des conidies (spores asexuées). Ces conidies se détachent du conidiophore et sont transportées par voie aérienne. Lorsqu'elles tombent sur une feuille, elles s'attachent sur l'épiderme de la plante. En présence d'eau libre, les spores germent au bout de 2h et forment un organe de pénétration, l'appressorium (8h), qui permet par pression de perforer la paroi végétale (8-12h) et de produire un hyphe qui se développe dans les cellules, causant des lésions visibles (5 j). Le champignon produit alors à nouveau des conidies qui seront dispersées à leur tour. Il existe aussi un cycle sexué qui n'a jamais été observé directement dans la nature mais qui est produit *in vitro*. *M. oryzae* est un champignon hétérothallique, ce qui signifie que la reproduction sexuée nécessite la mise en contact de deux souches de types sexuels opposés, Mat1 et Mat2. Au moins une des deux souches doit être femelle-fertile, c'est-à-dire capable de former les périthèces, organes femelles dans lesquelles sont produites les spores sexuées : les ascospores.

M. oryzae se développant sur les organes aériens, en particulier les feuilles, la pyriculariose diminue l'activité photosynthétique. Les dommages les plus importants sont causés par les attaques sur l'inflorescence (la panicule). Celles-ci entraînent une diminution du rendement pouvant aller jusqu'à 100% de pertes dans certaines conditions.

Le champignon est distribué dans toutes les zones de culture du riz dans le monde. La maladie a été détectée pour la première fois en Australie ce mois d'août 2011.

2. Histoire de la domestication du riz.

Le genre *Oryza* comprend 23 espèces, dont deux cultivées : *O. glaberrima* domestiqué et cultivé en Afrique et *O. sativa* cultivé sur tous les continents sauf l'Antarctique (Londo *et al.* 2006). *O. sativa* est essentiellement autogame. Le riz cultivé asiatique *O. sativa* a été domestiqué depuis son ancêtre sauvage *O. rufipogon.* Des preuves archéologiques de domestication ont été trouvées dans la vallée du Yangtze en Chine il y a 7000 ans (Fuller *et*

al. 2009). Sur la base du polymorphisme d'insertion du rétro-transposon *p-SINE1* (Short INterspersed Elements) entre *O. rufipogon* et *O. sativa*, Cheng *et al.* (2003) ont émis l'hypothèse de deux évènements indépendants de domestication du riz sauvage *O. rufipogon*, donnant lieu aux deux principales sous-espèces de riz cultivé : *O. sativa indica* à partir d'une lignée pérenne et *O. sativa japonica* à partir d'une lignée annuelle. Cette hypothèse est appuyée par les études de Yamanaka *et al.* (2003) et Londo *et al.* (2006). Londo *et al.* (2006) ont confirmé cette hypothèse par des analyses de réseaux d'haplotypes sur un gène chloroplastique et deux gènes nucléaires. Ils ont suggéré la domestication d'*O. sativa indica* dans une zone s'étendant de l'est de l'Inde à la Thaïlande et la domestication d'*O. sativa japonica* dans le sud de la Chine. Des allèles liés à la domestication, communs au deux sous-espèces, suggèrent des introgressions entre les deux pools domestiqués (Kovach *et al.* 2007).

Beaucoup plus tard, la révolution verte a donné lieu à de nombreux échanges de matériel végétal pour la création de variétés, et a conduit à une homogénéisation des cultivars utilisés en riziculture irriguée en zone tropicale. Deux cultivars en particulier, IR36 et IR64, ont été cultivés sur des millions d'hectares (Khush 2001). Pendant la révolution verte, IR36 est devenue une des variétés les plus cultivées (11 million ha/an) avant d'être remplacée par IR64 (Khush 2001).

3. Apparition de *M. oryzae* sur le riz domestiqué.

Les mécanismes d'apparition de la pyriculariose sur le riz cultivé *Oryza sativa* –c'est-à-dire l'apparition de souches pathogènes du riz de *M. oryzae*– n'ont pas encore été identifiés ni datés. Couch *et al*. (2005), à partir d'une étude phylogénétique, ont émis l'hypothèse selon laquelle *M. oryzae* serait apparu sur le riz par un saut d'hôte unique depuis une plante du genre *Setaria* (sétaires). Ce saut d'hôte serait contemporain à la domestication du riz par l'Homme. En effet, la domestication des sétaires serait contemporaine à la domestication du riz et aurait aussi eu lieu dans la vallée de la Yangtze en Chine (Hiroo *et al.* 2007). Le nombre de marqueurs SNPs (2 informatifs) et la taille de l'échantillonnage de souches sétaires ne permettent pas d'exclure complètement d'autres hypothèses, comme par exemple plusieurs sauts d'hôtes indépendants à partir de sétaires ou saut(s) d'hôte(s) à partir d'autres espèces. L'hypothèse d'un saut d'hôte unique est cependant soutenue par des études montrant que l'acquisition de la pathogénicité de *M. oryzae* sur le riz était un évènement évolutif unique

(Hamer et *al.* 1989 ; Borromeo *et al.* 1993 ; Shull & Hamer 1994). Stukenbrock & Mc Donald (2008) ont émis l'hypothèse alternative d'une domestication de *M. oryzae* en même temps que le riz (*host-tracking*).

V. Objectifs de la thèse

Chez *M. oryzae* le centre d'origine présumé sur le riz est le piémont himalayen (Chine, Népal, Inde, Laos, Vietnam) puisque la plus grande diversité génétique y a été observée (Tharreau *et al.* 2009). Or, le centre d'origine d'un agent pathogène peut être différent de son centre de diversité, du fait de flux de gènes importants et de grandes tailles de population pouvant générer une diversité génétique plus importante dans une aire introduite que dans l'aire d'origine (Stukenbrock & Mc Donald 2008 ; Gladieux *et al.* 2010).

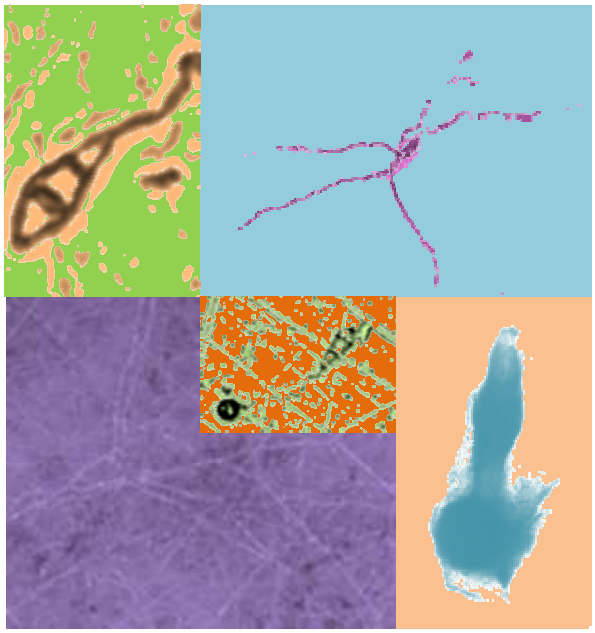
Le premier objectif de la thèse a donc été de tester l'hypothèse d'un centre d'origine de *M. oryzae* sur le riz cultivé correspondant au centre de diversité et au centre de dispersion en Asie. Il constitue le chapitre I de cette thèse, dans lequel un article en préparation sera présenté.

Nous avons également vu que le mode de reproduction d'un agent pathogène était susceptible d'avoir été modifié au cours de la domestication, de l'intensification et de la dispersion. Chez *M. oryzae*, la reproduction sexuée n'a jamais été observée directement dans la nature mais est inductible *in vitro.* Jusqu'à aujourd'hui, les conditions biologiques nécessaires à la reproduction sexuée (souches des deux types sexuels et souches femelle-fertiles) n'ont été observées qu'en Asie.

Le deuxième objectif de cette thèse a été de confirmer la présence de reproduction sexuée de *M. oryzae* sur le riz en Asie et son absence dans le reste du monde et de tester la possibilité de perte de reproduction sexuée avec la dispersion du champignon depuis l'Asie. Il constitue le chapitre II de cette thèse, où deux articles soumis pour publication dans Molecular Ecology et dans BMC Evolutionary Biology seront présentés.

CHAPITRE 1

Centre d'origine et dispersion de *Magnaporthe oryzae.*



I. Introduction

Nous avons vu en introduction qu'il existe différents mécanismes d'apparition des agents pathogènes sur les plantes domestiquées tels que le *host-tracking*, le saut d'hôte ou encore le transfert horizontal de matériel génétique. Afin d'anticiper l'évolution des agents pathogènes de plantes cultivées, il est nécessaire de comprendre les évènements passés ayant façonné la diversité génétique observée actuellement. Dans ce but, quatre questions se posent :

Quel est le centre de domestication de l'hôte ?

Quel est le centre d'origine de l'agent pathogène, c'est-à-dire son aire d'apparition sur l'hôte domestiqué ? La connaissance du centre d'origine permet de comprendre les conditions de l'émergence de l'agent pathogène.

Quel est le centre de diversité de l'agent pathogène, c'est-à-dire l'aire où la plus forte diversité génétique est observée ?

Quel est le centre de dispersion de l'agent pathogène, c'est-à-dire quelle est l'aire à partir de laquelle les plus fortes migrations sont parties ? La connaissance du centre de dispersion permet de comparer la structuration de la diversité génétique entre l'aire d'origine et les aires d'introduction, et donc de préciser les mouvements migratoires du pathogène au cours de sa dissémination –qui est souvent parallèle à celle de son hôte.

L'étude d'une invasion biologique repose souvent sur la comparaison de la population dans l'aire introduite avec la population dans l'aire native. Dans cette partie, nous nous proposons de faire une revue des mécanismes qui sont susceptibles de se produire dans le centre d'origine, le centre de diversité et le centre de dispersion, et de leurs conséquences sur la diversité génétique observée.

II. Distinctions entre le centre d'origine, le centre de diversité et le centre de dispersion.

Plusieurs études ont montré que le centre d'origine de l'agent pathogène correspond souvent au centre de domestication de son hôte (Stukenbrock & McDonald 2008). Chez les champignons par exemple, Gladieux *et al.* (2008) à partir d'une étude mondiale sur 12

marqueurs microsatellites, ont suggéré l'origine de *Venturia inaequalis,* agent de la tavelure du pommier mondialement distribué, en Asie Centrale. Gomes-Alpizar *et al.* (2007) ont suggéré une origine de *Phytophtora infestans,* responsable du mildiou de la pomme de terre, dans le centre de domestication de son hôte : les Andes.

Mais il existe des exemples d'agent pathogènes ayant émergé en dehors de la zone de domestication de leur hôte. C'est en effet le cas du champignon pathogène de l'orge, *Rynchosporium secalis.* A partir d'une étude phylogénétique utilisant un gène d'avirulence (*NIP1*), Brunner *et al.* (2007) ont montré que l'émergence de *R. secalis* sur l'orge n'était pas contemporaine à la domestication de son hôte dans le Croissant Fertile, mais était plus récente et se situait en Europe du Nord (Scandinavie).

Ainsi, même s'il est plus parcimonieux de faire l'hypothèse que le centre d'origine d'un agent pathogène coïncide avec le centre de domestication de son hôte, une hypothèse alternative peut être envisagée.

1. Centre d'origine et centre de diversité.

Souvent, l'expansion et la diversification d'un agent pathogène a lieu avant la dispersion (Figure 2.1a, b et c). Navarro & Barton (2003), à partir de réseaux d'haplotypes construits à partir de marqueurs mitochondriaux sur *Plasmodium falciparum*, agent responsable de la malaria, ont confirmé une expansion rapide dans son centre d'origine en Afrique concordant avec l'émergence des sociétés agricoles et la spéciation de son vecteur, *Anopheles gambiae*, sur l'Homme. *P. falciparum* a ensuite migré vers l'Asie, l'Amérique du Sud et la Papouasie Nouvelle Guinée.

L'introduction d'un agent pathogène dans une nouvelle aire implique souvent un faible nombre d'individus. Ceci a pour conséquences des goulots d'étranglement importants. Aussi peut-on s'attendre à observer une diversité génétique moins importante dans des zones introduites par rapport au centre d'origine d'une espèce. C'est en effet le cas pour un certain nombre d'espèces invasives. Par exemple, à partir d'une revue sur 80 espèces d'animaux, de végétaux et de champignons, Dlugosh & Parker (2008a) ont trouvé que la moitié de ces espèces affichaient une diminution de la diversité allélique de plus de 20% dans les aires envahies. Ces mêmes auteurs ont mis en évidence une diminution de 50% de la diversité allélique et de la proportion de loci polymorphes sur 189 marqueurs (séquences d'ITS et

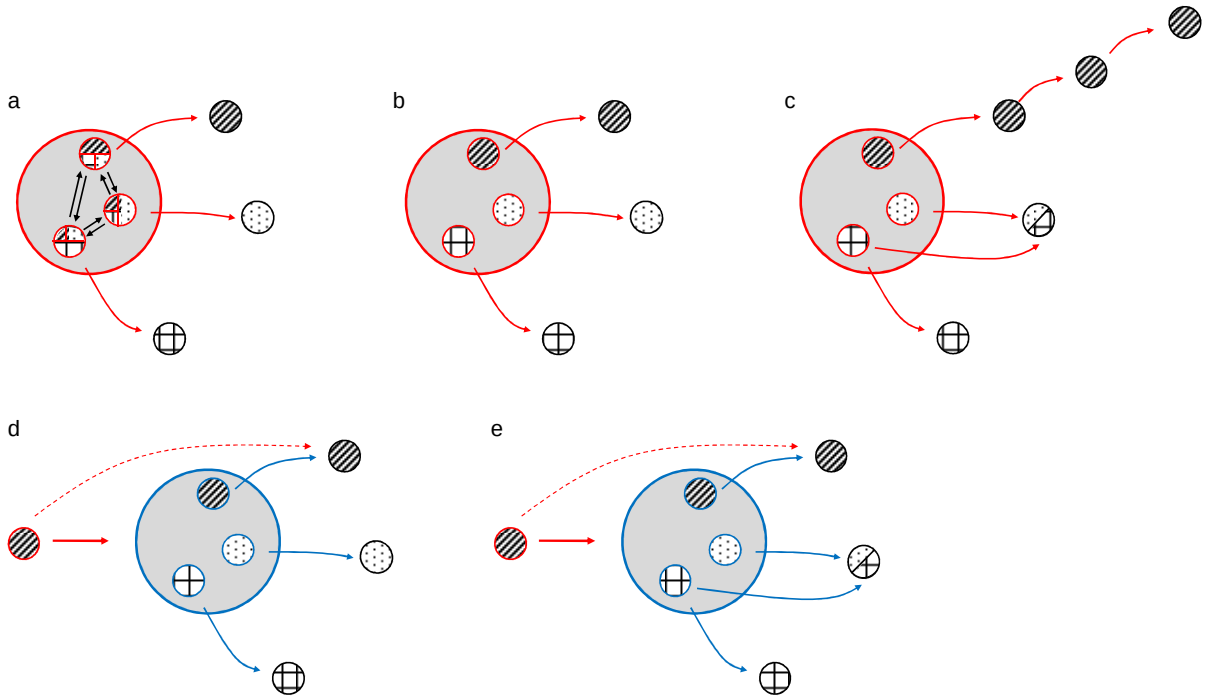


Figure 2.1. Quelques cas de figure sur l'origine, la diversification et la dispersion d'une espèce.
Les petits cercles représentent des populations. Les grands cercles représentent le centre d'origine en rouge et le centre de dispersion en bleu. Le centre de diversification est schématisé par un remplissage gris.
a. Le centre d'origine de l'espèce correspond au centre de diversification et comprend plusieurs populations qui échangent des migrants. C'est aussi le centre de dispersion puisque de chaque population partent des flux de migrants vers d'autres régions. La diversité génétique intra région et intra population la plus forte est détectée dans le centre d'origine.
b. Le centre d'origine de l'espèce correspond toujours au centre de diversification et de dispersion et comprend plusieurs populations différenciées. La diversité génétique intra région maximale est détectée dans le centre d'origine mais la diversité intra population y est faible.
c. Le centre d'origine correspond toujours au centre de diversification et de dispersion mais il existe des populations dans les zones introduites qui ont reçu des migrants depuis différentes populations de ce centre. La diversité génétique intra région est détectée dans le centre d'origine mais la diversité génétique intra population la plus forte est détectée dans une aire d'introduction. La population hachurée illustre le phénomène de tête de pont.
d. Le centre d'origine est différent du centre de diversification et de dispersion. La diversité génétique intra région la plus forte n'est plus détectée dans le centre d'origine.
e. Le centre d'origine est encore différent du centre de diversification et de dispersion. Certaines populations des zones introduites ont reçu des migrants depuis différentes populations du centre de diversification. La diversité génétique intra région la plus forte est détectée dans le centre de diversification et de dispersion et la diversité génétique intra population la plus forte est détectée dans une zone introduite.

AFLP) dans trois populations introduites d'une espèce de millepertuis, *Hypericum canariense*, par rapport à son aire native (Dlugosh & Parker 2008b). *Drosophila melanogaster* présente une diversité génétique plus importante dans son centre d'origine en Afrique que dans les aires d'introduction dans le reste du monde (Kauer *et al.* 2003). De même, Raboin *et al.* (2007), à partir d'une étude sur 17 marqueurs microsatellites ont mis en évidence une diversité génétique plus élevée du champignon pathogène de la canne à sucre *Ustilago scitaminea* dans son centre d'origine en Asie par rapport aux aires d'introduction en Afrique et en Amérique. Cette diminution de la diversité génétique dépend de plusieurs facteurs, en particulier la taille efficace de la population introduite et son taux de croissance (Nei *et al.* 1975). En effet, pour une petite taille efficace et un taux de croissance faible, une population est susceptible de perdre plus d'allèles, en particulier des allèles rares.

Cependant, il existe des cas où la diversité génétique n'est pas réduite après l'introduction d'une espèce dans une nouvelle aire. En effet, sur les 80 espèces de l'étude de Dlugosh & Parker (2008a), la moitié d'entre elles présente une diminution assez faible (inférieure à 20%) de la diversité génétique entre aire d'origine et aire introduite.

Le maintien de la diversité génétique en dehors de l'aire native d'une espèce peut s'expliquer par des introductions multiples (Keller & Taylor 2008 ; Whitney & Gabler 2008). C'est particulièrement vrai quand il existe une forte variabilité inter-populations et une faible variabilité intra-populations dans l'aire native. En effet, des introductions multiples dans une même aire à partir de populations différenciées dans l'aire native permettent d'augmenter non seulement la taille efficace et le taux de croissance dans la population introduite, mais aussi la diversité génétique (Figure 2.1d et e). Les introductions multiples ne s'accompagnent en revanche pas systématiquement d'un mélange des différents pools génétiques. Un exemple illustrant cette situation est celui de *Phytophthora infestans*, agent du mildiou de la pomme de terre, célèbre pour la grande famine qu'il déclencha dans les années 1850 en Irlande (Goodwin *et al.* 1994). Cet agent pathogène a été introduit de multiples fois en Europe depuis le XIXe siècle mais il existe deux populations en sympatrie en France, provenant de deux évènements d'introduction, qui sont complètement différenciées (Montarry *et al.* 2010). Une des deux populations était déjà établie quand la seconde a été introduite et elles ne se sont pas mélangées du fait d'une reproduction clonale très importante. C'est aussi le cas chez *Cryphonectria parasitica*, champignon pathogène du châtaignier, dont l'origine est en Amérique du Nord. Trois groupes génétiques ont en effet détectés en France correspondant à

des évènements d'introduction différents (Dutech *et al.* 2010). La faible intensité de flux de gènes entre les différentes populations et la faible expansion de la maladie en France (majoritairement dans le sud) pourrait s'expliquer par la fragmentation des habitats de l'hôte dans la zone introduite. Des introductions multiples de *C. parasitica* ont aussi été suggérées en Espagne sur la base de la présence de différents types de compatibilité végétative (González-Varela *et al.* 2011).

Le maintien de la diversité génétique peut aussi être expliqué par la vitesse d'expansion de la population introduite. En effet, les tailles de populations d'agents pathogènes peuvent augmenter rapidement dans une population d'hôte homogène de grande taille, ce qui permet le maintien ou même l'augmentation de la diversité génétique. Chez *V. inaequalis*, Gladieux *et al.* (2008) ont identifié un goulot d'étranglement fort au moment de l'introduction de l'agent pathogène en Europe, et ont pourtant observé le maintien d'une diversité génétique importante dans les aires introduites. Ils expliquent ce patron par des introductions multiples, des flux de gènes intra-régionaux importants, et l'expansion importante des populations introduites grâce à l'intensification des vergers de pommiers dans les aires introduites.

Ainsi, le centre d'origine d'un agent pathogène sur une plante donnée ne correspond pas systématiquement à l'aire géographique où se trouve la diversité génétique la plus importante. On peut donc différencier centre d'origine et centre de diversité.

2. Mouvements de migration : centre d'origine et centre de dispersion.

Dlugosh & Parker (2008a) ont fait l'observation que, pour étudier les changements génétiques liés à l'introduction d'une espèce invasive dans une nouvelle aire, il est indispensable de déterminer le plus précisément possible la source dans l'aire native, c'est à dire de comprendre à quelle(s) population(s) de l'aire native cette population introduite se rattache. Les changements évolutifs attribués aux évènements d'introduction peuvent en fait découler d'évènements ayant eu lieu dans la zone source. Inférer la source précise d'une introduction nécessite donc de déterminer l'échelle à laquelle les populations sont

différenciées dans l'aire native. Ainsi, les échantillons collectés doivent être représentatifs de la diversité génétique globale dans cette aire et les marqueurs utilisés doivent avoir le pouvoir de résolution adapté (Dlugosh & Parker, 2008a).

De plus, le centre d'origine d'une espèce ne correspond pas systématiquement à la zone depuis laquelle les populations ont été dispersées. En d'autres termes, le centre de dispersion ne correspond pas forcément au centre d'origine, ni au centre de diversité d'une espèce. C'est particulièrement vrai dans le cas des agents pathogènes d'espèces domestiquées, dont les migrations sont souvent fortement inféodées aux mouvements de populations humaines. Par exemple, chez *Rynchosporium secalis,* champignon pathogène de l'orge, Brunner *et al.* (2007) ont montré des taux de migrations importants depuis des centres secondaires en Europe de l'Ouest (Royaume-Uni, Allemagne et Suisse). Les populations d'Australie proviendraient essentiellement du Royaume-Uni et d'Allemagne et les populations du Croissant Fertile proviendraient de Suisse (Figure 2.2). Pour *R. secalis*, le centre d'origine en Scandinavie n'est donc pas le seul centre de dispersion (Figure 2.3). De la même manière, chez *V. inequalis*, des taux de migration importants ont été détectés non seulement depuis le centre d'origine, mais aussi entre différentes aires d'introduction. L'Europe, en particulier est considérée comme un centre de dispersion et un centre de diversité secondaire du champignon puisqu'elle présente une diversité génétique comparable à celle de l'Asie Centrale et plus importante que celle observée dans les aires d'introduction plus récentes en Amérique et en Afrique (Gladieux *et al.* 2008). C'est d'ailleurs depuis l'Europe que se fait l'essentiel des migrations vers ces régions. Ainsi, le centre d'origine de *V. inequalis* est bien en Asie mais le centre de dispersion est en Europe. Banke & McDonald (2005) ont estimé les taux de migration entre différentes populations de *Mycosphaerella graminicola*, champignon pathogène du blé, dont l'origine est contemporaine à la domestication de son hôte au Moyen-Orient. Ils ont mis en évidence des taux de migration vers l'Amérique et l'Australie plus importants depuis l'Europe que depuis le Moyen-Orient. L'Europe n'est donc pas le centre d'origine de *M. graminicola,* mais en est un centre de dispersion important.

Il existe aussi des mécanismes « tête de pont » lorsqu'un agent pathogène est introduit dans une nouvelle aire, il peut se disperser progressivement pour former une population continue (Figure 2.1c)

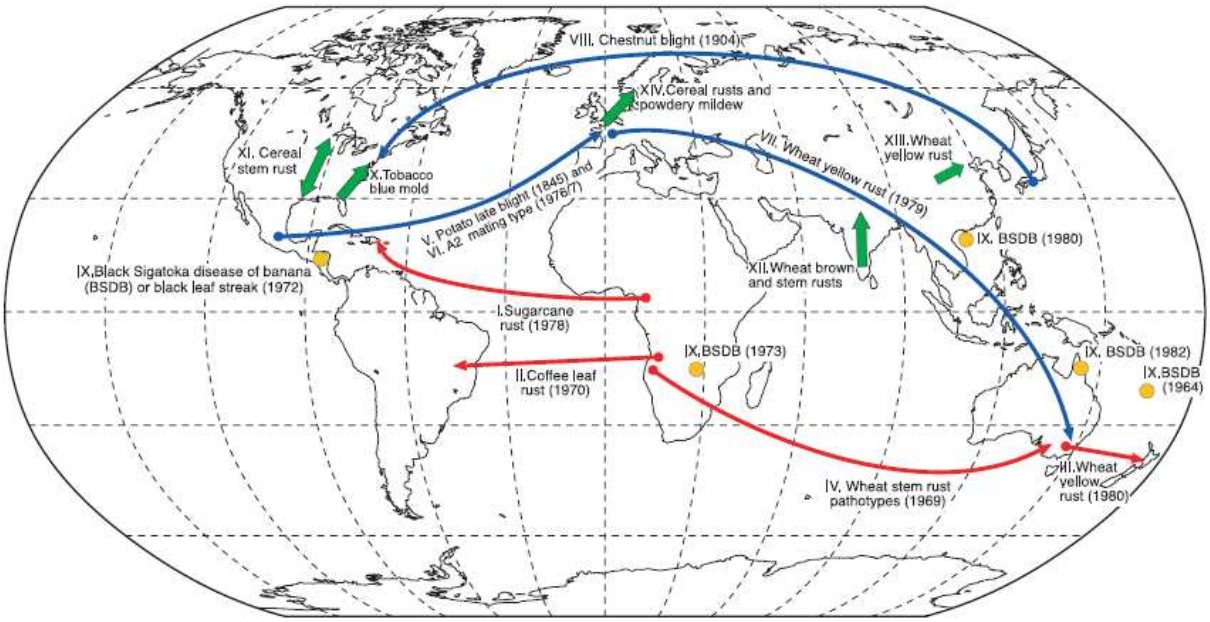


Figure 2.2. Exemples de routes de dispersion chez 14 champignons phytopathogènes. (d'après Brown & Hovmoller, 2011)

Les flèches indiquent l'invasion d'une nouvelle aire et la date de première détection est précisée entre parenthèses.

Les flèches rouges indiquent une dispersion naturelle de spores par le vent :
I : *Puccinia melanocephala* sur canne à sucre,
II : *Hemileia vastatrix* sur caféier,
III : *Puccinia striiformis f. sp. tritici* sur blé,
IV : *Puccinia graminis f. sp. tritici* sur blé.

Les flèches bleues indiquent une dispersion par le transport de matériel infecté par l'Homme.
V et VI : *Phytophtora infestans* sur pomme de terre,
VII : *Puccinia striiformis f. sp. tritici* sur blé,
VIII : *Cryphonectria parasitica* sur marronnier.

Les cercles orange indiquent la dispersion de *Mycosphaerella fijiensis* sur bananier (IX).

Les flèches vertes indiquent des migrations périodiques de spores véhiculées par le vent dans des cycles d'extinction-recolonisation.
X : *Peronospora tabacina* sur tabac,
XI : *Puccinia graminis f. sp. tritici* sur blé,
XII : *Puccinia graminis f. sp. tritici* et *P. triticina* sur blé,
XIII : *Puccinia striiformis f. sp. tritici* sur blé,
XIV : rouilles et mildious des céréales.

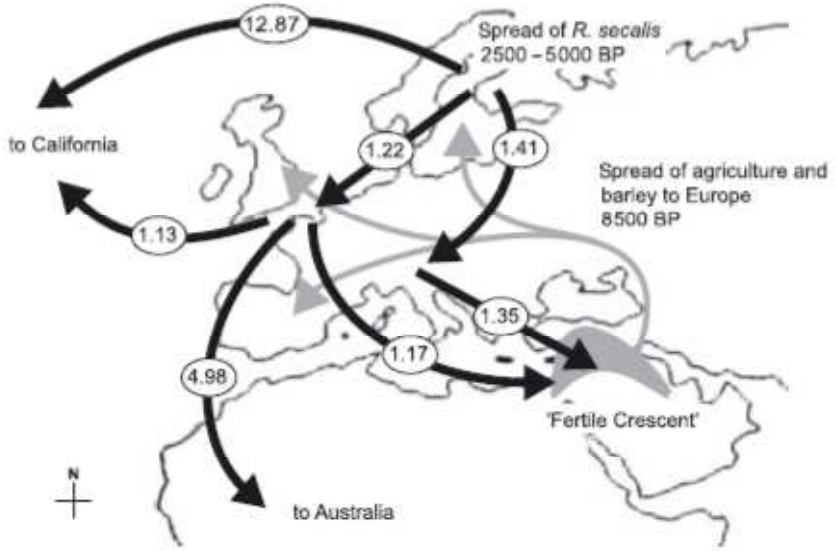


Figure 2.3. Principales routes de dispersion de *Rynchosporium secalis* depuis l'Europe vers le Moyen-Orient, l'Amérique du Nord et l'Australie.
(d'après Brunner *et al.*, 2007)
Les flèches noires et grises représentent respectivement les flux de migration de *R. secalis* et les routes de migration des fermiers du Néolithique vers l'Europe.

Ainsi, le centre d'origine d'une espèce peut être différent non seulement du centre de diversité mais aussi du centre de dispersion, ou au moins peut ne pas être le seul centre de diversité et de dispersion.

3. Modes de dispersion chez les champignons phytopathogènes : dispersion naturelle ou humaine ?

Afin de déterminer la part de migrations naturelles et la part de migrations provoquées par l'Homme, il est nécessaire d'inférer les origines et les routes de dispersion d'un agent pathogène. Les données historiques permettent de compléter ces informations. Les flux de gènes des agents pathogènes dépendent très fortement des flux de gènes des hôtes (Criscione 2008). Cela est particulièrement vrai chez les agents pathogènes de plantes cultivées dont la dispersion s'effectue essentiellement par le transport de matériel végétal infecté. Palm (2001) a recensé quelques uns des champignons phytopathogènes introduits par l'Homme en Amérique du Nord par le transport de matériel végétal infecté, comme *Puccinia graminis* sur le blé ou encore *Discula destructiva* sur le cornouiller. De même, l'agent du charbon de la canne à sucre *Ustilago Scitaminea*, a été introduit depuis l'Asie vers l'Afrique et l'Amérique par du matériel infecté (Raboin *et al.* 2007). La mise en place de quarantaines au cours de ces cinquante dernières années n'a pas permis de bloquer complètement l'expansion des agents pathogènes de cultures (Wingfield *et al.* 2001 ; Brown & Hovmoller 2011). L'homogénéisation des cultivars modernes à l'échelle mondiale favorise la dispersion des maladies de plantes par l'échange de matériel végétal infecté et l'expansion après introduction. Cependant, il existe aussi des exemples de champignons phytopathogènes, en particulier ceux responsables des rouilles, dont la dispersion n'est pas provoquée uniquement par l'Homme au travers des échanges de matériel infecté, mais aussi par le transport de spores par le vent (Brown & Hovmoller 2011). Brown & Hovmoller (2011) suggèrent que, bien que rares, les évènements de dispersion à pas unique (*single-step*) à longue distance ont des conséquences importantes en termes de dynamique des populations.

4. Description de la structure génétique des populations.

Finalement, dans l'étude des champignons phytopathogènes (et plus généralement des espèces envahissantes), il est primordial de bien différencier trois centres qui peuvent être bien distincts : le centre d'origine, le (ou les) centre(s) de diversification et le (ou les) centre(s) de dispersion (Figure 2.1).

Dans l'étude de l'origine d'un organisme et de sa dispersion, les indices de génétique des populations sont couramment utilisés pour estimer la diversité génétique dans les différentes aires de répartition. La richesse allélique, mesurée comme le nombre moyen d'allèles par locus étudié, permet d'estimer de manière correcte les effets d'un goulot d'étranglement, par la comparaison des valeurs entre différentes populations (Leberg 2002). Cette approche peut dans certains cas être biaisée par les tailles d'échantillons mais reste très informative (Figure 2.4). Les méthodes d'assignation représentent un outil complémentaire pour étudier la structure des populations, la dispersion, et l'hybridation (Manel *et al.* 2005). Enfin, il existe des méthodes permettant d'inférer des scenarii de dispersion en testant différentes valeurs de paramètres comme les tailles efficaces et les taux de migration. Ces méthodes récentes d'*Approximate Bayesian Computation* (ABC) sont susceptibles d'améliorer la compréhension des migrations d'agents pathogènes.

III. Origine et dispersion de *M. oryzae*.

L'Asie est le centre de domestication du riz, hôte domestiqué de *M. oryzae* (Fuller *et al.* 2009). L'hypothèse de centre d'origine de *M. oryzae* sur le riz cultivé dans l'aire de domestication a été émise par Couch *et al.* (2005). Nous avons voulu, dans cette partie, tester cette hypothèse par des méthodes de génétique des populations. Par ailleurs, la plus forte diversité génétique a été détectée en Asie (Tharreau *et al.* 2009). Nous avons testé l'hypothèse de centre de diversité de *M. oryzae* en Asie. Enfin, nous avons cherché à déterminer l'origine précise des populations introduites dans le monde. Pour déterminer les trois centres (origine, diversité et dispersion) de *M. oryzae*, nous avons réalisé des analyses de génétique des populations afin de déterminer la structure et la différenciation des populations à l'échelle de l'Asie du Sud-Est et à l'échelle mondiale.

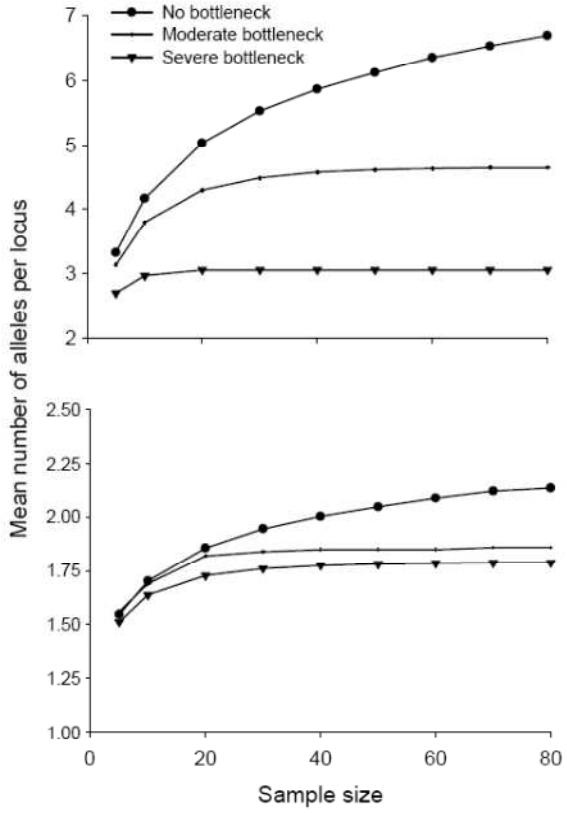


Figure 2.4. Richesses alléliques (nombre moyen d'allèles par locus) pour différentes tailles d'échantillons calculés sur 1000 populations simulées par combinaison de conditions.
(d'après Leberg, 2002)
Les graphiques du haut et du bas représentent des populations avec des niveaux de polymorphismes respectivement élevés et bas. Les populations représentées par des cercles n'ont pas été exposées à un goulot d'étranglement (500 individus) les autres ont été exposées à un goulot d'étranglement modéré (tirets : réduction à 24 individus) ou sévère (triangles : réduction à 8 individus).

IV. Article 1. South-East Asia is the centre of origin, the centre of diversity and the centre of dispersion of the fungus pathogenic on rice, *Magnaporthe oryzae.*

Les travaux réalisés dans ce chapitre sont présentés sous la forme d'un article, qui est en cours de corrections pour être soumis.

South-East Asia is the centre of origin, the centre of diversity and the centre of dispersion of the fungus pathogenic on rice, *Magnaporthe oryzae*.

Dounia Saleh, Joelle Milazzo, Henri Adreit, Elisabeth Fournier and Didier Tharreau.

Introduction

Magnaporthe oryzae is an Ascomycete fungus responsible for the most damaging fungal disease on rice worldwide: blast. It is considered as a model for the study of host-pathogen interaction (Valent 1990; Talbot 2003; Ebbole 2007). The center of origin of *M. oryzae* on cultivated rice is still unresolved. As for many pathogens, it might correspond to the center of domestication of its host, *Oryza sativa*. The history of *O. sativa* is relatively well understood. There have been two independent domestication events of wild rice *O. rufipogon* ~7000 years BP, resulting in two sub-species: *O. sativa indica* domesticated south of the Himalayas (likely eastern India, Myanmar or Thailand) and *O. sativa japonica* domesticated in southern China (Cheng *et al.* 2003; Londo *et al.* 2006; Fuller *et al.* 2009). These regions are also the centers of diversification of rice (Londo *et al.* 2006). From a phylogenetic analysis on *M. oryzae* strains from ten different genera of host plants, Couch *et al.* (2005) suggested a single origin of strains pathogenic on rice due to a host shift from strains pathogenic on foxtail millets (*Setaria spp*). This hypothesis was supported by two SNPs (Single Nucleotide Polymorphism). The authors suggested that the center of origin of *M. oryzae* on rice was in China, since both rice and foxtail millet were domesticated and co-cultivated there. Shull & Hamer (1994) also suggested a single origin of *M. oryzae* on cultivated rice because of a single acquisition of pathogenicity. So, as the origin of domesticated rice is in Asia, the hypothesis of origin of *M. oryzae* on rice in this region is not unlikely. However, the center of origin of a plant disease does not always colocalize with the center of domestication of its host (Brunner *et al*. 2007). Therefore, alternative hypotheses cannot be ruled out.

Another observation reinforcing the hypothesis of Asia as the center of origin of *M. oryzae* on rice, is the fact that female-fertile strains were observed only in this area. Leslie & Klein (1996) suggested that several Ascomycete fungal species that have lost the ability to reproduce sexually in introduced areas may still be able of sexual reproduction in the center of origin. In the case of *M. oryza,* the reproductive mode is mainly asexual (Zeigler 1998) and sexual reproduction has never been detected directly in the field. However, sexual

reproduction has been achieved *in vitro* and requires two strains of opposite mating types (Mat1 and Mat2) comprising at least one female-fertile strain, that is, able to produce the sexual organs where meiosis takes place (perithecia). Female-fertile strains were found exclusively in Asian countries (Zeigler 1998; Kumar *et al.* 1999) and the two mating types are present in Asia. On the contrary, only one mating type is found in some other regions in the world. Tharreau *et al.* (2009), using multivariate analysis on 13 microsatellite markers, indeed found a worldwide genetic structure in three populations: one population gathered only Mat1 strains, one population gathered only Mat2 strains and the third population grouped strains of the two mating types from Asia.

Several studies described the diversity of genetic diversity of *M. oryzae* in Asia. Numerous lineages of *M. oryzae* were characterized in India (George *et al.* 1998; Kumar *et al.* 2009) and in Korea (Park *et al.* 2003 and 2008), that were tightly linked to rice cultivars. In the Philippines, although the genetic diversity was lower than in India (George *et al.* 1998), the genetic structure of *M. oryzae* lineages was linked to rice cultivars, especially to the subspecies *indica* and *japonica* (Chen *et al.* 1995; Zeigler *et al.* 1995)

On the contrary, in other parts of the world, there were few lineages and the structure was consistent with clonal reproduction. This was the case in Europe (Roumen *et al.* 1997; Piotti *et al.* 2005), in Iran (Javan-Nikkhah *et al.* 2004) and in North America (Levy *et al.* 1991; Xia *et al.* 1993 and 2000; Correll *et al.* 2009).

However few studies described the population structure of *M.* oryzae, at continental or global scales, except Zeigler (1998), Tharreau *et al.* (2009) and Faivre Rampant *et al.* (Annexe 1). Based on a review analysis of the genetic diversity in different countries, Zeigler (1998) concluded that the genetic diversity is higher in Asia compared to other regions. Tharreau *et al.* (2009) confirmed this result by analyzing the genetic diversity of a worldwide collection of strains. So, *M. oryzae* showed lower diversity out of Asia. This observation raises the question of localizing the origin of *M. oryzae* migrations: is this pattern the result of migrations from Asia to the rest of the world? In *M. oryzae*, Tharreau *et al.* (2009) studied the distribution and the polymorphism of the avirulence gene *ACE1* over all rice growing countries. *ACE1* is recognized by *Pi33*, a resistance gene present in semi-dwarf rice varieties developed during the green revolution in the 60's (Ballini *et al.* 2008). Tharreau *et al.* (2009) found that two virulent genotypes were widely distributed all over the world. They concluded that there should have been dissemination of these strains *via* infected plant material during the last 50 years. They also suggested that, in line with epidemiological results, spore

dispersal was limited between fields as they found significant differentiation between fields of a same location in France and in Madagascar. *M. oryzae* strains are thus more likely to disperse *via* the transport of infected materials at the global scale.

All these studies support the hypothesis that the origin of *M. oryzae* strains pathogenic on rice is in Asia, that most of the genetic diversity observed in the world is represented in this region, and that Asia may also be the centre from which the pathogen dispersed towards the rest of the world. The aim of this work was to test these hypotheses using a population genetics approach on populations of *M. oryzae* from different continents (Asia, Europe, Americas, and Africa) and from Madagascar. In the study of migration, a source population is expected to show higher genetic diversity than the sink populations founded from it (Gladieux *et al.* 2008). During the introduction of a species in a new area, demographic bottlenecks are indeed expected to decrease genetic diversity. We first compared the level of genetic diversity in Asia and in the other areas at the population scale, confirming the high genetic diversity in Asia. We then used clustering methods to describe the structure of the genetic diversity in Asia, in order to precise the area in Asia likely to be the center of diversity. Clustering methods were also used to describe the worldwide genetic structure, and to assign the genetic groups observed outside Asia to Asian populations, confirming that in *M. oryzae*, the center of diversity coincides with the center of dispersion. Crosses were performed on strains from some of these populations to determine the distribution of mating types in the world and to verify that the conditions in which sexual reproduction can occur are met only in Asia, reinforcing the idea that Asia is also the center of origin of the disease.

Material and Methods

Samples

For this study we used 55 samples of *M. oryzae* strains isolated from cultivated rice throughout the world mainly between 2000 and 2009, representing 1372 strains in total (Table 3.1). A sample was composed of strains collected in the same field on the same variety or on similar varieties. In some cases, different samples were collected at the same place but at different years; we grouped them as a single population after having verified that they were not genetically differentiated based on F_{ST} estimated from microsatellite markers. This was the case for CH1 and MD1 samples. The Thai sample was the only one that was not isolated

Area	Country	population	N	type of culture	mating type	Female fertility	$H_{n.b.}$	N_a	N_p	*MLG*	*G:N*	$\bar{r}_D$
Asia	China	CH1	107	upland rice	39 Mat1 61 Mat2 7 nd	187 ff 13 fs 7 nd	0.629	6.9	1.6	82	77%	0.069
-	-	CH2	38	irrigated	17 Mat1 21 Mat2	4 ff 34 fs	0.522	3.4	0.2	21	55%	0.292
-	-	CH3	23	upland rice	14 Mat1 9 Mat2	5 ff 18 fs	0.499	3.7	0.1	11	48%	0.189
-	-	CH4	25	upland rice	25 Mat1 0 Mat2	1 ff 24 fs	0.051	1.3	0	4	16%	0.209
-	-	CH5	28	upland rice	13 Mat1 14 Mat2 1 nd	0 ff 27 fs 1 nd	0.541	3.8	0	19	68%	0.094
-	-	CH6	30	irrigated	26 Mat1 4 Mat2	0 ff 30 fs	0.253	2.6	0	7	23%	0.368
-	-	CH7	14	irrigated	2 Mat1 8 Mat2 4 nd	0 ff 5 fs 9 nd	0.256	2.1	0	9	64%	0.150
-	Indonesia	ID1	20	irrigated	1 Mat1 10 Mat2 9 nd	1 ff 0 fs 19 nd	0.277	2.3	0.1	10	50%	0.135
-	-	ID2	19	irrigated	0 Mat1 9 Mat2 10 nd	nd	0.089	1.4	0.1	4	21%	0.095
-	-	ID3	16	upland rice	0 Mat1 6 Mat2 10 nd	nd	0.114	1.7	0	6	38%	0.053
-	Laos	LA1	15	upland rice	9 Mat1 5 Mat2 1 nd	0 ff 14 fs 1 nd	0.569	3.4	0.4	12	80%	0.154
-	-	LA2	9	upland rice	5 Mat1 4 Mat2	6 ff 3 fs	0.518	3.5	0.2	8	89%	0.073
-	Nepal	NP1	31	irrigated	5 Mat1 1 Mat2 25 nd	0 ff 6 fs 25 nd	0.168	2.6	0.1	11	35%	0.295
-	-	NP2	15	upland rice	4 Mat1 7 Mat2 4 nd	2 ff 9 fs 4 nd	0.403	2.8	0.3	6	40%	0.330
-	-	NP3	6	semi-irrigated	4 Mat1 0 Mat2 2 nd	0 ff 4 fs 2 nd	0.491	2.4	0.1	4	67%	0.651
-	Thailand	TH	27	-	6 Mat1 21 Mat2	17 ff 10 fs	0.460	3.6	0.1	18	67%	0.166

Area	Country	population	N	type of culture	mating type	Female fertility	$H_{n.b.}$	N_a	N_p	*MLG*	*G:N*	$\bar{r}_D$
Europe / Mediterranean basin	France	FR1	23	irrigated	23 Mat1 0 Mat2	0 ff 23 fs	0.124	1.7	0	9	39%	0.050
	-	FR2	17	irrigated	nd	nd	0.163	1.9	0	9	53%	-0.010
-	-	FR3	18	irrigated	nd	nd	0.112	1.6	0	6	33%	0.113
-	-	FR4	17	irrigated	nd	nd	0.110	1.6	0	8	47%	0.009
-	-	FR5	22	irrigated	nd	nd	0.019	1.2	0.1	3	14%	-0.036
-	-	FR6	37	irrigated	nd	nd	0.317	2.3	0.1	8	22%	0.398
-	-	FR7	15	irrigated	nd	nd	0.024	1.1	0	2	13%	nd
-	Grece	GR1	10	irrigated	7 Mat1 0 Mat2 3 nd	0 ff 7 fs 3 nd	0.246	1.7	0	6	60%	0.261
-	-	GR2	10	irrigated	3 Mat1 0 Mat2 6 nd	0 ff 4 fs 6 nd	0.183	1.8	0	6	60%	0.144
-	-	GR3	10	irrigated	8 Mat1 0 Mat2 2 nd	0 ff 8 fs 2 nd	0.225	1.7	0	4	40%	0.456
-	-	GR4	9	irrigated	7 Mat1 0 Mat2 2 nd	0 ff 9 fs	0.102	1.5	0	3	33%	0.801
-	-	GR5	9	irrigated	9 Mat1 0 Mat2	0 ff 9 fs	0.167	1.6	0	3	33%	0.436
-	-	GR6	10	irrigated	10 Mat1 0 Mat2	0 ff 10 fs	0.190	2	0	4	40%	0.544
-	-	GR7	9	irrigated	7 Mat1 0 Mat2 2 nd	0 ff 7 fs 2 nd	0.115	1.3	0	3	33%	0.791
-	Hungary	HN1	7	irrigated	nd	nd	0.070	1.2	0	3	43%	0.452
-	-	HN2	3	irrigated	nd	nd	0.053	1.1	0	2	67%	nd
-	-	HN3	7	irrigated	nd	nd	0.101	1.3	0	4	57%	-0.055
-	Marocco	MC	15	irrigated	10 Mat1 0 Mat2 5 nd	0 ff 9 fs 6 nd	0.169	1.9	0.1	8	53%	0.097

Area	Country	population	N	type of culture	mating type	Female fertility	$H_{n.b.}$	N_a	N_p	*MLG*	*G:N*	$\bar{r}_D$
Europe / Mediterranean basin	Spain	SP1	12	irrigated	3 Mat1 1 Mat2 8 nd	0 ff 1 fs 11 nd	0.260	2	0	6	50%	0.344
	-	SP2	31	irrigated	5 Mat1 0 Mat2 26 nd	nd	0.204	2.1	0	11	35%	0.245
-	-	SP3	11	irrigated	nd	nd	0.258	1.7	0	5	45%	0.545
-	-	SP4	22	irrigated	5 Mat1 0 Mat2 17 nd	nd	0.278	2.1	0	9	41%	0.399
-	-	SP5	13	irrigated	nd	nd	0.039	1.2	0	3	23%	nd
-	-	SP6	9	irrigated	nd	nd	0.052	1.1	0	2	22%	nd
-	-	SP7	10	irrigated	nd	nd	0.044	1.1	0	2	20%	nd
-	-	SP8	18	irrigated	nd	nd	0.052	1.1	0	2	11%	nd
-	-	SP9	29	irrigated	nd	nd	0.057	1.3	0.1	4	14%	-0.046
-	Turkey	TR	19	irrigated	nd	nd	0.048	1.3	0	4	21%	-0.079
America	Colombia	CL1	17	upland rice	nd	nd	0.083	1.4	0	2	12%	0.217
-	-	CL2	31	upland rice	0 Mat1 31 Mat2	0 ff 31 fs	0.052	1.4	0.1	5	16%	-0.057
-	French Guyana	GY	12	irrigated	0 Mat1 4 Mat2 8 nd	nd	0.219	1.9	0.2	3	25%	0.805
-	USA	USA1	37	irrigated	10 Mat1 24 Mat2 4 nd	0 ff 37 fs	0.558	3.2	0.1	14	38%	0.469
-	-	USA2	39	irrigated	29 Mat1 0 Mat2 10 nd	nd	0.018	1.3	0	3	8%	0.280
Madagascar	Madagascar	MD1	264	upland rice	0 Mat1 142 Mat2 122 nd	0 ff 108 fs 246 nd	0.039	3.7	0.3	24	9%	0.191
-	-	MD2	27	irrigated	0 Mat1 19 Mat2 8 nd	nd	0.169	2	0	10	37%	0.047
-	-	MD3	37	upland rice	0 Mat1 32 Mat2 5 nd	0 ff 1 fs 36 nd	0.115	2.2	0	12	32%	0.061
-	-	MD4	15	upland rice	0 Mat1 2 Mat2 13 nd	nd	0.000	1	0	1	7%	nd
-	-	MD5	23	irrigated	0 Mat1 21 Mat2 2 nd	0 ff 1 fs 22 nd	0.193	2.4	0	11	48%	0.022
-	-	MD6	25	upland rice	0 Mat1 15 Mat2 10 nd	nd	0.074	1.7	0	7	28%	-0.007

Table 3.1. Information on the 55 samples.
Origin, sample name, samples size (*N*), type of culture, proportion of Mat1 and Mat2 strains, proportion of female-fertile and female-sterile strains, unbiased gene diversity ($H_{n.b.}$), mean number of alleles per locus (N_a), number of multilocus genotypes (*MLG*), clonal richness (*G:N*), mean number of private alleles per locus (N_p) and multilocus linkage disequilirium ($\bar{r}_D$). nd: not determined either because it was not tested or because the test was not reliable. ff: female-fertile. fs: female-sterile. Some $\bar{r}_D$ could not be calculated because of *MLGs* that were not shared by enough individuals.

from rice as it was obtained from a barley seed lot in 1987. However, barley is a broad spectrum host to *M. oryzae* and this sample shared the genetic and pathogenic characteristics of *M. oryzae* strains isolated from rice and not to other barley strains (D. Tharreau personal communication). This sample came therefore from a "rice" population. Altogether, these 55 samples represented all continents but did not contain West African populations.

Strain isolation and storage

For fungal strain isolation, infected plant material was placed in humid chamber at 25°C during 1-2 days. After monospore isolation, strains were grown on rice flour medium (rice flour 20g; yeast extract 2g; agar 15g; water 1L and Penicillin G: 500,000 UI added after autoclaving 20min at 120°C) as previously described by Silué & Nottéghem (1990). Strains were stored as described by Valent *et al.* (1986). They were grown on a filter paper disk overlaying the rice flour medium. The filter paper colonized by the fungus was then dried at 35°C for 4-5 days, cut into pieces and distributed in sterile paper bags. Paper bags were placed in plastic bags sealed under vacuum and stored at -20°C.

Determination of mating type and fertility

For about half of the strains, especially in Asian samples, mating type and female-fertility were determined by *in vitro* crosses described by Nottéghem & Silué & Nottéghem (1992). *M. oryzae* is a heterothallic fungus with two mating types (Mat1 and Mat2) that are controlled by two idiomorphs. A strain can only mate with a strain of opposite mating type. In addition, for sexual reproduction to occur between two strains, at least one of them must be female-fertile, that is able to produce perithecia-the female organs were meiosis takes place. Crosses were performed by confronting the strain to test to reference strains on culture medium, a reference strain being a female-fertile strain for which the mating type is known. Each strain was tested with two Mat1 and two Mat2 reference strains. If no perithecia were produced, the tested strain had the same mating type as the reference strain. If perithecia were produced the tested strain was of opposite mating type to the reference strain. In this case, if only one line of perithecia was observed on the confrontation line between the tested strains and the reference strain, then the tested strain was female-sterile. On the contrary, if two lines of perithecia were observed, the tested strain was female-fertile. Some strains were completely sterile, that is they could not reproduce sexually even with a strain of opposite mating type and female-sterile. Mat1 reference strains were IN1, TH12, CH999 and CH1003. Mat2 reference strains were GY11, TH16, CH997 and CH1019. For some strains, mating type

was determined by PCR amplification with the primers specific of Mat1.1 and of Mat1.2 developed by Xu & Hamer (1995). In that case, female fertility could not be assessed.

DNA extraction and microsatellites amplification

DNA extraction was performed as described by Adreit *et al.* (2007), following a CIAA procedure. All strains were genotyped with 10 microsatellites markers (Table 3.2) previously developed (Kaye *et al.* 2003; Adreit *et al.* 2007). Amplifications were performed using the QIAGEN multiplex PCR kit (15min at 94°C; 1min at 94°C, 30sec at 57°C, 1min30 at 72°C for 30 cycles and 30min 60°C). After amplification, one µl of each PCR product was mixed with 15µl of Formamide GeneScan-500LIZ size marker (Applied Biosystems, Foster City, CA) and analyzed on a 16-capillary ABI Prism 3130XL (Applied Biosystems). Allele calling was achieved using GENEMAPPER® software (Applied Biosystems).

Indices of genetic diversity in populations

For each population, the mean number of alleles per locus (N_a) and the unbiased gene diversity $H_{n.b.}$(Nei 1978) were calculated using GENETIX software (Belkhir *et al.* 2004). We also calculated N_p, the mean number of private allele per locus in each population, corresponding to the number of alleles averaged over all loci that were present only in one population. The number of multilocus genotypes (MLG) and the index of association $\overline{r}_D$ were calculated using MULTILOCUS 1.3 software (Agapow & Burt 2001). $\overline{r}_D$ is an association index based on the variance of the distance between all pairs of individuals. It differs from the I_A estimate of multilocus linkage disequilibrium as it is corrected for the number of scored loci. By construction, $\overline{r}_D$ is expected to tend towards zero in panmictic populations and towards one if the whole genome is in linkage disequilibrium. The proportion of MLG in each sample was calculated as the ratio G:N, G being the number of MLG and N the sample size.

Clustering and assignment analyses

Before using clustering methods with prior assignment to geographic populations we estimated pairwise F_{ST} (Weir & Cockerham 1984) implemented in the software GENEPOP 4 (Raymond & Rousset 1995).

Marker name	Chromosome	Supercontig (Range on Supercontig pb)	Repeat motif	Primers sequences	T_m (°C)	N_t
pyrms63-64	1	9 (243439-243601)	CT_{15}	F: (NED)-TTGGGATCTTCGGTAAGACG R: GCCGACAAGACACTGAATGA	57	13
pyrms83-84b	2	18 (211631-211742)	TCA_{12}	F: (PET)-GTCTGCCTCGACTCCTTCAC R: GCAAAGTTGTTTGAGCAAGG	57	10
pyrms319-320	2	18 (52505-52795)	CAA_6	F: (NED)-TAAGACCACTGGCGGAATCT R: GGCTTTGTCTGGTTGTACGG	57	6
pyrms77b-78b	3	24 (510594-510813)	CA_{24}	F: (PET)-AGGCTCTCTGCCTACGAAGT R: GCTTTCGGCAAGCCTAATC	57	22
pyrms607-608	3	28 (1095575-1095864)	GCA_{13}	F: (VIC)-CCCAAGCTCCATAATACGCTAC R: TCCGAGACTCTTTGGATAGCAC	57	16
pyrms37-38	4	15 (1042756-1042961)	CA_7+CT_{12}	F: (NED)-ACCCTACCCCCACTCATTTC R: AGGATCAGCCAATGCCAAGT	57	8
pyrms47-48	4	12 (707029-707203)	TA_{15}	F: (FAM)-TCACATTTGCTTGCTGGAGT R: AGACAGGGTTGACGGCTGAA	57	13
pyrms233-234	5	10 (38361-38617)	CAG_{10}	F: (FAM)-TGAGATGGACCGCATGATTA R: TTGATGGCAGAGACATGAGC	57	14
pyrms427-428	5	13 (331827-332045)	AT_{16}	F: (VIC)-CTGTCACCACAACCAAGACG R: TTGCCCTGATTTGTCAGTCA	57	22
pyrms657-658	6	21 (3745780-3745947)	CA_{12}	F: (VIC)-ATCAGTCGAACCCACAAAGC R: ATGTGTGGACGAACCAGTCC	57	6

Table 3.2. Characteristics of the ten microsatellite markers used in this study. Name, position, repeat motif, primers sequences, melting temperature (T_m), and total number of alleles over the 1372 individuals.

Two complementary methods were used to estimate the optimal number of clusters present in the Asian dataset (all samples from China, Indonesia, Laos, Nepal and Thailand) and in the complete dataset. We first used the Bayesian method implemented in the software STRUCTURE (Pritchard *et al.* 2000; Falush *et al.* 2003). This method divides a dataset into a number of clusters *K* by minimizing linkage disequilibrium (or maximizing Hardy-Weinberg equilibrium) within clusters. It applies to populations that are panmictic. The basic assumptions underlying this method are that organisms are sexually reproducing, and that the analyzed sample can be theoretically divided into panmictic clusters. However, the method is robust to departure from panmixia hypothesis in a dataset and give relevant results also in clonal or autogamous organisms. We used the model with correlated allele frequencies and allowing admixture. There is admixture when individuals can have mixed ancestry, and have equivalent probabilities to belong to several clusters. For each run, Markov Chain Monte Carlo (MCMC) of 80,000 steps was performed after a burn-in period of 20,000. We did not give any prior on individual's assignments. For the analysis of the Asian dataset, STRUCTURE was run for *K* ranging from 1 to 32. For the analysis of the complete dataset, it was run for *K* ranging from 1 to 37. We performed 10 replicates for each value of K. For each analysis, to determine the optimal number of clusters, we used the method developed by Evanno *et al.* (2005) based on the rate of change in the log probability of data (provided by STRUCTURE) between successive *K* values. Individuals were assigned to a cluster if their posterior probability to belong to this cluster was higher than 0.7.

Since *M. oryzae* is essentially clonal (even if recombination was found is Asian populations, see Saleh et al.), this organism does comply with the basic assumptions of the STRUCTURE approach. Therefore, we used a second type of method, namely the Discriminant Analysis of Principal Components (DAPC) developed by Jombart *et al.* (2010). Unlike the STRUCTURE method, this multivariate method does not require any assumption on the biology of the organism. It consists in a Discriminant Analysis on data transformed after a Principal Component Analysis. The DAPC was conducted using the *adegenet* package (1.3-1) for the R software (2.13.1). We used the K-means procedure implemented in the function *find.cluster* to determine the optimal number of clusters *K*. *K* varied between 1 and 60 and the optimal value of K was determined using the Bayesian Information Criterion (BIC): it corresponds to the value of K for which the BIC is minimal, or at which the rate of change in the BIC abruptly changes (broken line).

Clone correction

Clustering analyses using STRUCTURE and DAPC were repeated on clone corrected data. When several individuals shared the same MLG within a sample, only one individual was kept. Clone corrected data set encompassed 649 individuals comprising 298 Asian individuals.

Source of variation within clusters

Chi^2 tests were performed to test if cluster assignment was independent from the type of culture (irrigated rice or upland rice), the mating type (Mat1 and Mat2) and female-fertility status (female-fertile or female-sterile).

Isolation by distance

The distribution of individuals in a region can be represented in a lattice and constitute a continuous population if individuals migration between the different point is not negligible. If dispersal is restricted in space, the individuals that are close geographically should be close genetically and on the contrary individuals that are isolated geographically should be differentiated genetically. To determine how strains dispersal was shaped in Asia, we performed isolation by distance (IBD) tests between pairs of samples, as implemented in GENEPOP (Raymond & Rousset, 1995). This method gives an estimate of the "neighborhood size" (the product of density D and the second moment of dispersal distance σ^2) and performs the linear regression of genetic distance $F_{ST}/(1-F_{ST})$ to the logarithm of geographic distance (Rousset, 2000). Mantel permutation test was performed to test the hypothesis that this regression slope was different from zero (Leblois *et al*. 2003), using 1000 permutations. We used GPS coordinates for each Asian sample and the approximate coordinates were assessed from the name of the location from which the strains were sampled when the exact GPS coordinates were not available.

In Europe/Mediterranean Basin, we had 28 samples that were distributed in six countries. In four of them, we had at least three samples. So the test of IBD was also performed in this region to determine whether genetic differentiation was higher between geographically isolated countries than between close countries. In Americas and in

Madagascar, the number of samples –two in the North America, two in the South America and six in Madagascar- was not enough to test IBD.

Population tree

We calculated Cavalli-Sforza distance between all sample pairs and constructed a tree using the software POPULATIONS using the neighbour-joining method. We also constructed a tree based on the F_{ST} between sample pairs. The software DARWIN was used to visualize the trees.

Results

Genetic diversity and linkage disequilibrium

The mean number of alleles per locus (N_a), the number of private alleles (N_p), and the unbiased gene diversity ($H_{n.b.}$) were higher in samples from Asia than in the other samples (Table 3.1). In Asia, Europe/Mediteranean Basin, America and Madagascar, mean $H_{n.b.}$ values were 0.38±0.19 SD, 0.14±0.09, 0.19±0.22 and 0.10±0.07 respectively, mean N_a values were 3.0±1.3, 1.6±0.4, 1.8±0.8 and 2.2±0.9 respectively, and mean N_p values were 0.19±0.38, 0.01±0.04, 0.08±0.08 and 0.05±0.12 respectively. The lowest mean values of $H_{n.b}$, N_a and N_p among Asian countries were in Indonesia (0.16±0.10, 1.8±0.5 and 0.07±0.06 respectively).

In Asia, the averaged proportion of multilocus genotypes (MLG) in samples (*G:N*) was 54%±23% SD (China: 50%±23%; Indonesia: 36%±15%; Laos: 84%±6%; Nepal: 57%±24% and Thailand: 67%). It was lower in Europe/Mediterranean Basin region (37%±16%), in America (20%±12%) and in Madagascar (27%±16%). This can be explained on the one hand by the higher allelic diversity in Asia, and on the other hand by the existence of recombination in Asian samples. The mean values of $\overline{r_D}$ in Asia, Europe/Mediterranean Basin, Americas and Madagascar were 0.21±0.15 SD, 0.27±0.27, 0.34±0.32 and 0.06±0.08 respectively. Multilocus linkage is expected to tend to zero in panmictic populations and to one in clonal populations. However, this index also strongly depends on several factors such as effective population size and migration rate (DeMeeûs & Balloux 2004). A population that results from multiple introductions from different gene pools is expected to exhibit a high level of multilocus linkage disequilibrium. As alleles at different loci may be different between the different sources, the whole genome indeed should be in linkage disequilibrium between the different gene pools and a greater number of MLG than that expected under

sexual reproduction mode should be observed. The MLG showed that the USA1 sample was composed of two differentiated clones, explaining why we observed higher gene, allelic and genotypic diversity than in sample USA2.

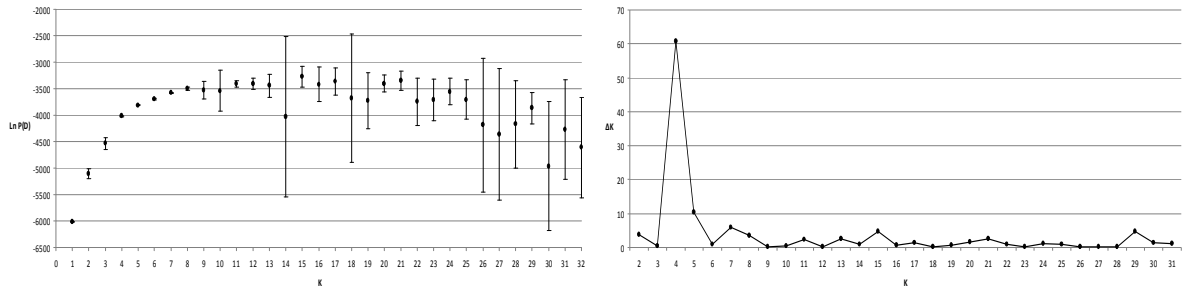
Clustering/assignment analyses in Asia

As we found the highest genetic diversity in Asia, which is the center of domestication of rice in Asia (Zeigler 1998), we looked at the distribution of genetic diversity inside Asia to find putative regions in Asia likely to be centers of diversity. The analysis without prior assignment to geographic samples and using STRUCTURE revealed that the genetic diversity in Asia was organized in four clusters (Figure 3.1a). The repeatability of the ten replicates was complete as individual assignments to the four clusters did not fluctuate at all among replicates. Over the 423 Asian individuals, 390 could be assigned to a single cluster (Figure 3.1b). Another analysis was conducted by incorporating a geographic prior to samples. Assignment was slightly enhanced since this second analysis yielded a higher value of the log probability of data (Ln p(D)= -3985.78) compared to the analysis without prior (Ln p(D)= -4020.99). The model with geographic priors exactly confirmed the four clusters.

An identical structure was found using DAPC. We used the function *find.cluster* to estimate the Bayesian Information Criterion (BIC) for a number of clusters ranging from 1 to 60. Theoretically, the optimal number of clusters is the one that presents the lowest BIC. In our study, the BIC did not reach a minimum (Figure 3.2a). In this case, the optimal value of *K* is the value after which the BIC changes by a negligible amount (Jombart *et al.* 2010). So, we plotted the mean difference between successive BIC values of *K*, $\Delta BIC = BIC(K) - BIC(K-1)$ and retained the value of *K* after which ΔBIC did not increase drastically. Again, we obtained *K*=4 (Figure 3.2a). For the Discriminant Analysis (DA), we retained 40 Principal Components (PCs), that allowed explaining >90% of the variance observed. The assignments of individuals to the four clusters were identical to those obtained with STRUCTURE with the exception of 12 individuals that showed admixture between cluster 1 and 4 (six individuals from CH1, three individuals from LA1 and three individuals from TH). The DAPC also achieved to assign the 40 individuals that were not assigned in the STRUCTURE analysis. Figure 3.2b represents the individuals on the (1,2) plan.

The structure we found could not be strictly correlated to geography. However, clusters and geographical origins were not independent (Figure 3.3). The samples CH1, LA1, LA2 and TH shared a similar structure with individual assigned essentially to cluster 4 (red),

a



b

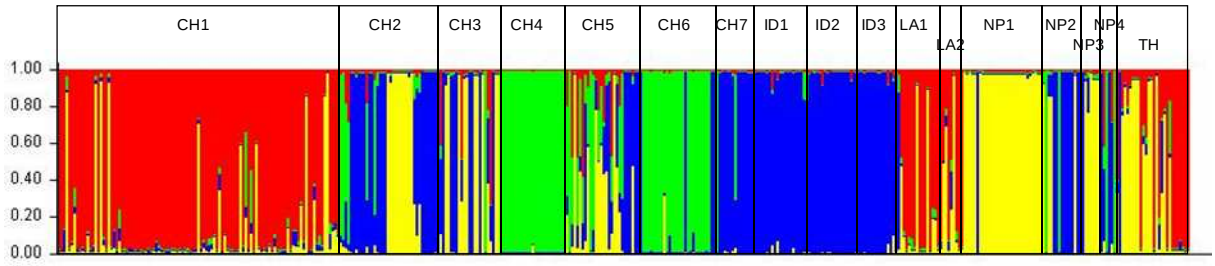
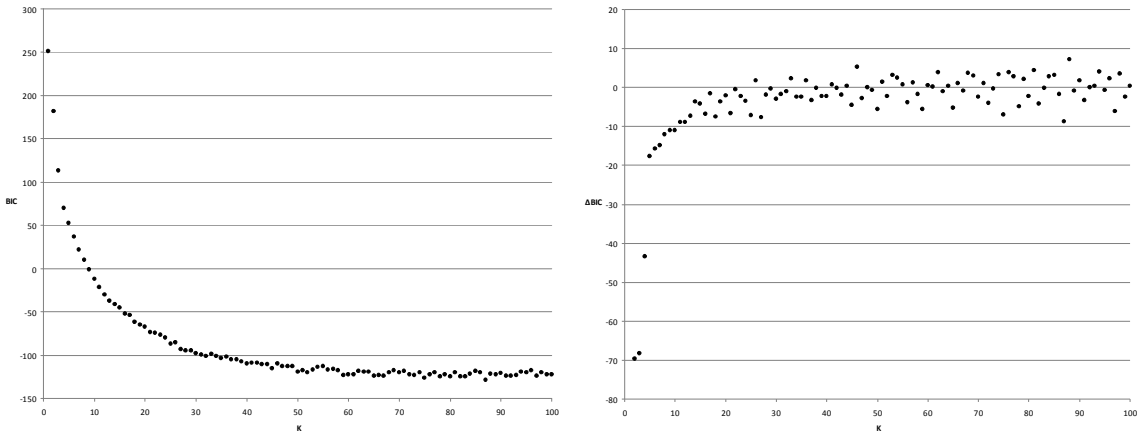


Figure 3.1. Genetic structure among the 423 Asian strains of *M. oryzae*, inferred by the software STRUCTURE, without geographic prior.
a. Determination of the number of clusters (K) using the rate of change in the log probability of data between successive *K* values (Evanno *et al.*, 2005). The rate of change in the log posterior probability of data picks for K=4, indicating that the explanatory value in describing the genetic structure is optimized for this value of K. **b.** Assignments of individuals to each of the four clusters (1-blue, 2-red, 3-green and 4-yellow). Each vertical bar represents an individual, and the posterior probability of assignment to a cluster is given by the length of the line of the corresponded colour. Admixed individuals are represented by two (or more) coloured lines.

a



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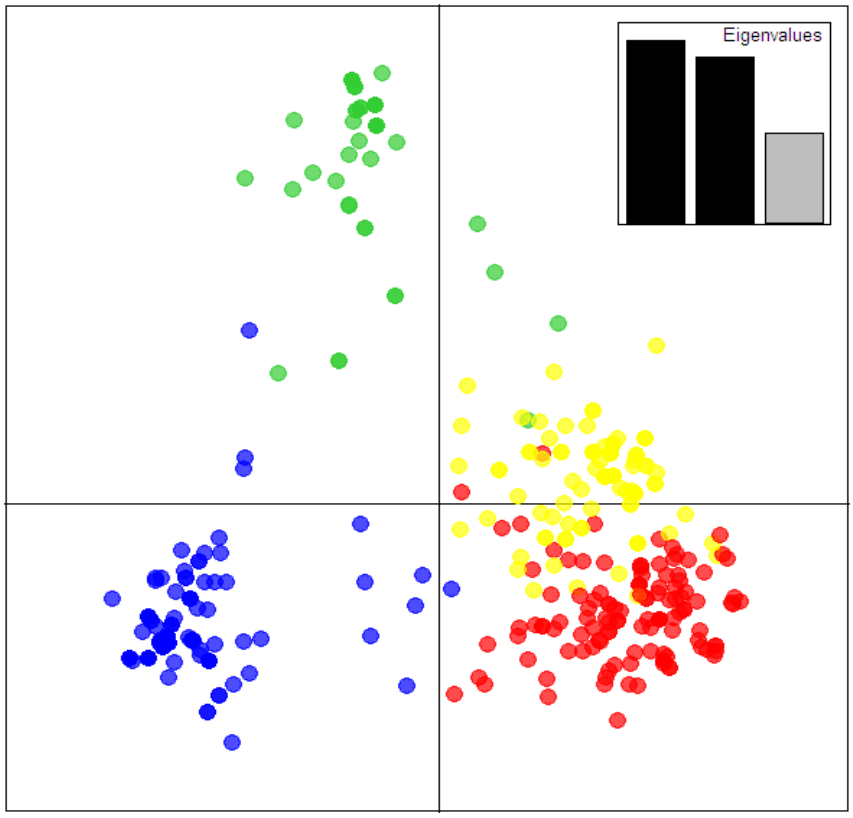


Figure 3.2. DAPC on the 423 Asian strains of *M. oryzae*.
a. Bayesian Information Criterion (BIC) as a function of number of clusters *K*. Difference between successive BIC values of *K*. **b.** Individuals represented in the (1,2) plan. The inset gives the eigenvalues of the DAPC.

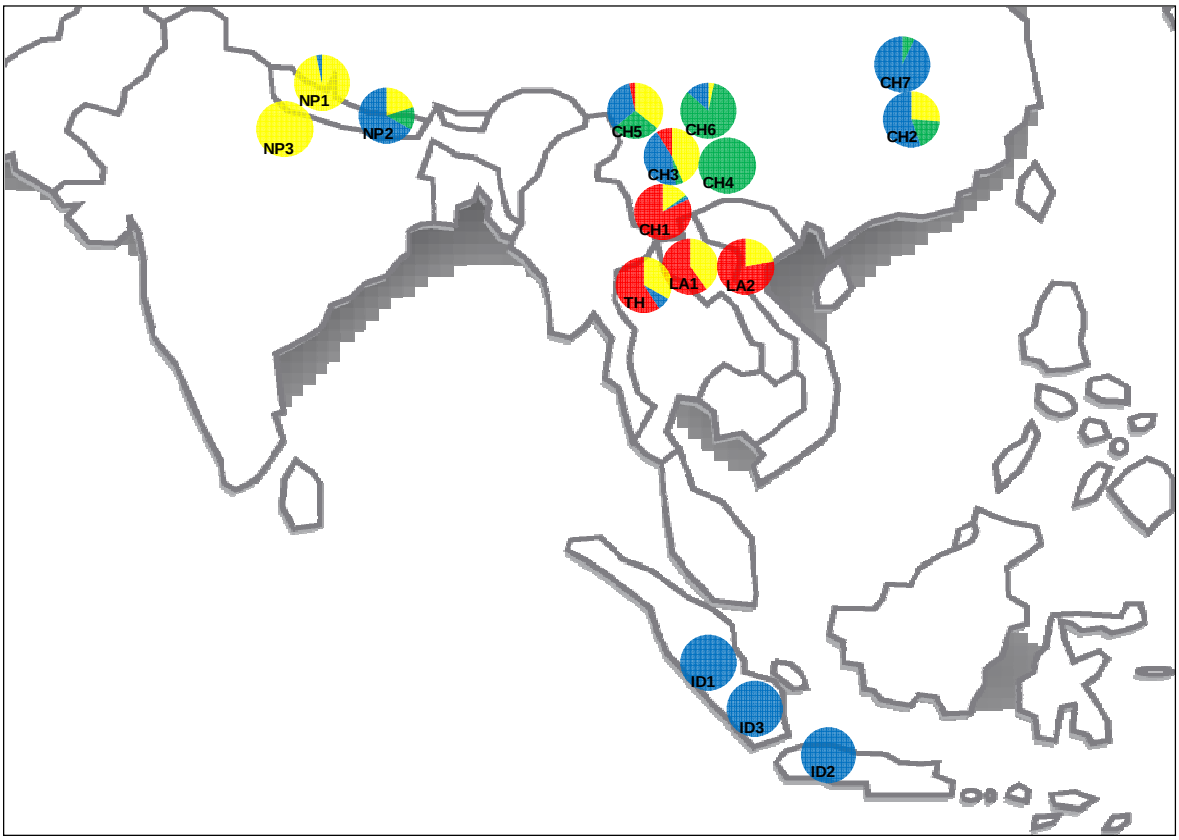


Figure 3.3. Proportion of strains belonging to the four clusters inferred using DAPC in 16 Asian samples.
The names of the samples are given in the piecharts. Cluster 1: yellow, cluster 2: green, cluster 3: blue and cluster 4: red.

cluster	*N*	$H_{n.b.}$	N_a	N_p	*MLG*	*G:N*	$\overline{r}_D$
1	103	0.504	7.9	0.19	55	53%	0.097
2	72	0.256	3.6	0.04	21	29%	0.182
3	131	0.259	5.5	0.03	49	37%	0.078
4	124	0.610	7.7	0.14	96	77%	0.030

Table 3.3. Genetic diversity within each of the four clusters of *M. oryzae* Asian strains inferred using DAPC. Cluster size (*N*), unbiased gene diversity ($H_{n.b.}$), mean number of alleles per locus (N_a), number of multilocus genotypes (*MLG*), clonal richness, (*G:N*), mean number of private alleles per locus (N_p) and multilocus linkage disequilirium ($\overline{r}_D$).

Fst	1	2	3
2	0.4352*		
3	0.4877*	0.6304*	
4	0.2668*	0.4752*	0.38*

Table 3.4. Pairwise F_{ST} between pairs of clusters.

*pairs significantly differentiated.

and to a lesser extent to cluster 1 (yellow). Samples CH2, CH3 and CH5 also showed a similar structure: individuals of these samples were essentially assigned to cluster 1 (yellow) and cluster 3 (blue). Interestingly, the two Chinese samples CH4 and CH6 were composed almost exclusively of individuals from cluster 2 (green). The sample CH7 and the Indonesian samples were composed exclusively of individuals from cluster 3 (blue). The Nepalese samples were divided into two groups: NP1 and NP3 encompassed individuals from cluster 1 (yellow) whereas NP2 was composed of individuals assigned essentially to cluster 3 (blue).

The clusters 1 (yellow) and 4 (red) had higher gene diversity than clusters 2 (green) and 3 (blue) ($H_{n.b}$: 0.50, 0.61, 0.26 and 0.26 respectively; Table 3.3). Clusters 1 and 4 had also higher allelic diversity (N_a 7.9 and 7.7 respectively) than clusters 2 and 3 (N_a: 3.6 and 5.5 respectively) and the mean number of private alleles per locus was also higher in clusters 1 and 4 (N_p: 1.9 and 1.4 respectively) than in clusters 2 and 3 (N_p: 0.4 and 0.3 respectively). The clonal richness was higher in clusters 1 and 4 (*G:N*: 53% and 77% respectively) than in clusters 2 and 3 (*G:N*: 29% and 37% respectively). So, clusters 1 and 4 showed higher genetic diversity than clusters 2 and 3. Each cluster was significantly differentiated from the others (genic differentiation, G log likelihood ratio tests, table 3.4) and F_{ST} were higher than 0.2 in all pairs of clusters. The lowest F_{ST} obtained was between clusters 1 and 4. We performed DAPC within each cluster, to find a putative sub-structure. The function *find.cluster* did converge towards an optimal *K* in cluster 2 and cluster 3. In cluster 1 and cluster 4, we found respectively four and nine clusters. However, the results could not be interpreted with regards to the geographic origin of samples or any other feature.

We performed a Mantel test between all the samples and between samples within the four clusters (Figure 3.4) to test the hypothesis of isolation by distance, and determine if the Asian samples formed a continuous population with stepping-stone migration from sample to sample. We did not find any significant IBD between samples over all clusters (P=0.07), within cluster 1 (P=0.11), within cluster 2 (P=0.68) and within cluster 4 (P=0.09). There was a slightly significant correlation within cluster 3 (P=0.04). However, in this latter case the slope between geographic distance and genetic distance was negative. So, for cluster 3, samples that were geographically closer were genetically more differentiated.

We also performed STRUCTURE and DAPC analyses on the clone corrected dataset. For *K*=4 we found the same assignations of individuals to the four clusters. However the repeatability between replicates was not as good.

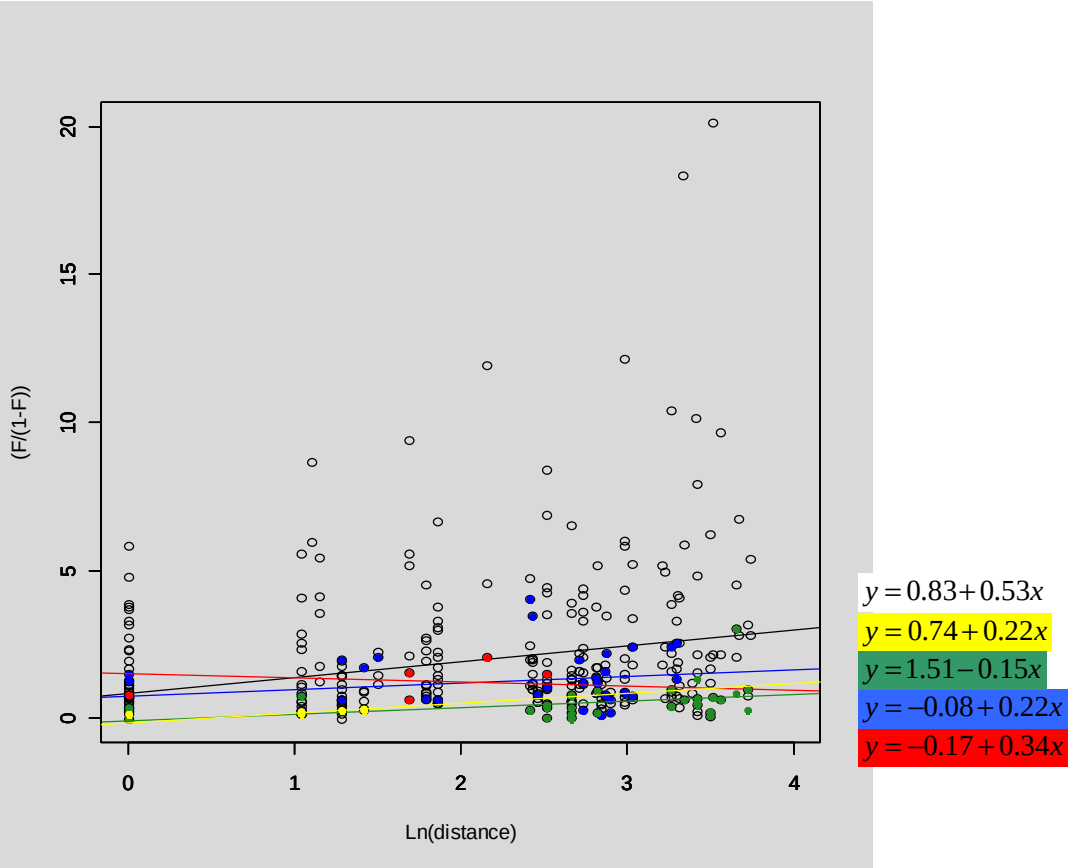


Figure 3.4. Genetic distance (F/(1-F)) as a function of geographic distance (Ln(distance)) between 16 *M. oryzae* Asian populations.
The regression equations are given for pairwise comparisons between all samples and between samples from the same cluster (cluster 1: yellow, cluster 2: green, cluster 3: blue, and cluster 4:red).

Clustering/assignment analyses in the complete dataset.

We looked if strains from samples out of Asia could be genetically related to the Asian clusters. For that, we performed again the DAPC on the 423 Asian strains and added the other strains as supplementary individuals using the function *pred.sup* implemented in the package *Adegenet* of the R software. This function allowed representing strains from out of Asia on a principal components plan without including them in the analysis (Figure 3.5). The European/Mediterranean strains were all but one assigned to cluster 2. The South American and the Malagasy strains were assigned to cluster 3. The North American strains were assigned to clusters 1, 2 and 4. We then conducted clustering analyses on the complete dataset to confirm this genetic structure.

The STRUCTURE analysis without geographic prior gave three main clusters (Figure 3.6a). The repeatability of the ten replicates was also perfect in this analysis. Over the 1372 individuals, only 16 showed admixture signals (comprising 14 Asian strains), and could therefore not be assigned to a single cluster (Figure 3.6b).

An identical structure was found using DAPC. We used the function *find.cluster* to estimate the BIC for a number of clusters ranging from 1 to 60. Again, the BIC did not reach a minimum (Figure 3.7a). The mean difference between successive BIC values of *K*, $BIC'(K)$ allowed us to retain the optimal number of clusters *K*=3 (Figure 3.7a). For the Discriminant Analysis (DA), we retained 40 Principal Components (PCs), that allowed explaining >90% of the variance observed. The DAPC achieved to assign the 16 individuals that were not assigned in the STRUCTURE analysis. The assignments of individuals to the three clusters were identical to those obtained with STRUCTURE with the exception of 4 individuals (one from CH5, one from TH and two from SP1). Figure 3.7b represent the individuals on the (1,2) plan.

When performed on a clone corrected dataset, STRUCTURE analysis and DAPC on the global dataset gave the same results.

Here, the structure was highly correlated to the geographic origin of the strains (Figure 3.8). The three clusters described at the worldwide scale were all represented in Asia. Cluster A (red) corresponded to the clusters 1 and 4 in the Asian analysis, cluster B (green) corresponded to cluster 2, and cluster C (blue) corresponded to cluster 3. Therefore, the 3-clusters structure depicted in Asia through the global analysis was in accordance with the 4-clusters structure obtained through the analysis of the Asian dataset alone. All individuals

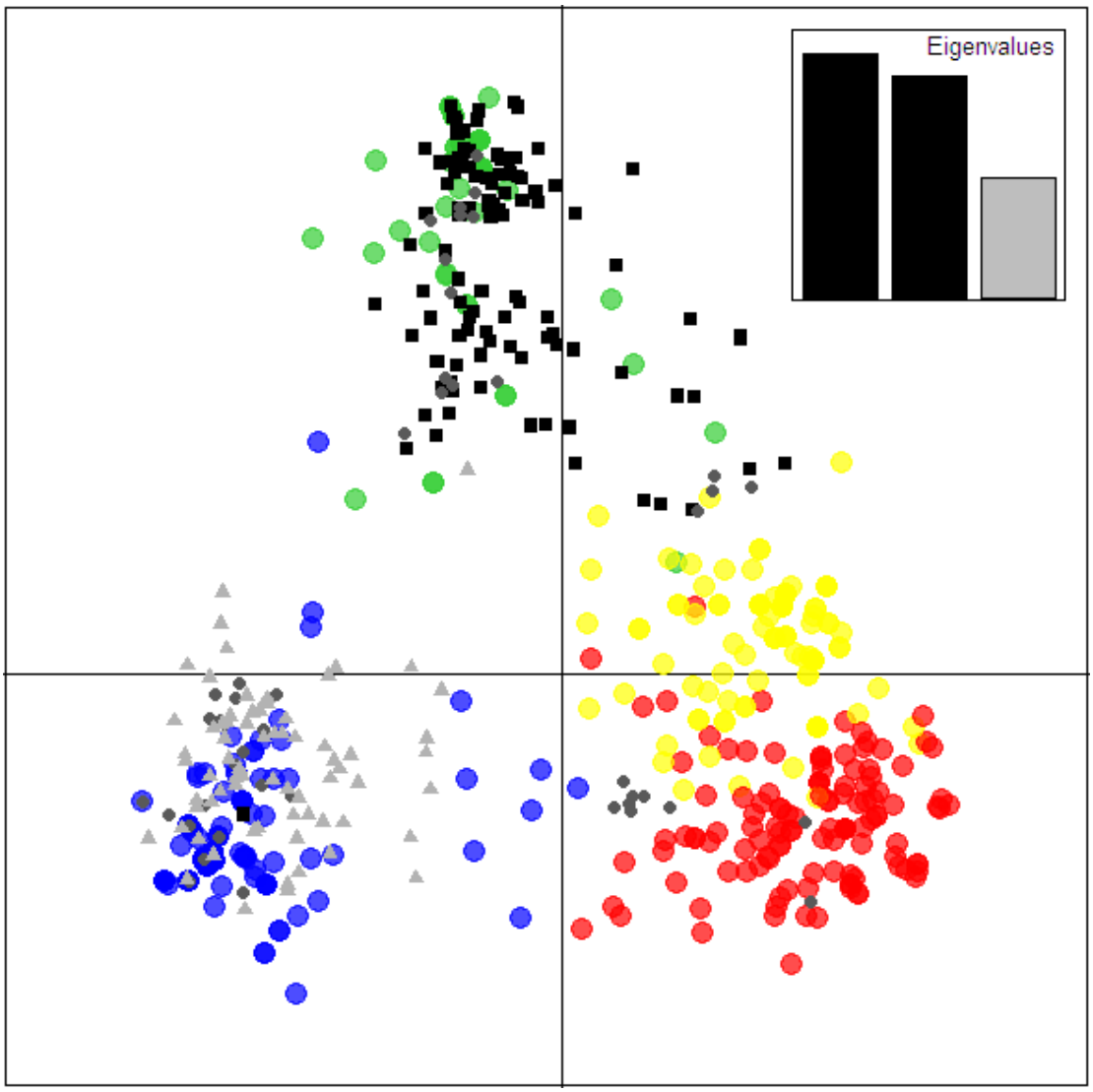


Figure 3.5. DAPC on the 423 Asian strains of *M. oryzae*.
Individuals are represented in the (1,2) plan with worldwide individuals from out of Asia as supplementary individuals (black squares: Europe/Mediterranean Basin, dark grey circles: America, light grey triangles: Madagascar). The inset gives the eigenvalues of the DAPC.

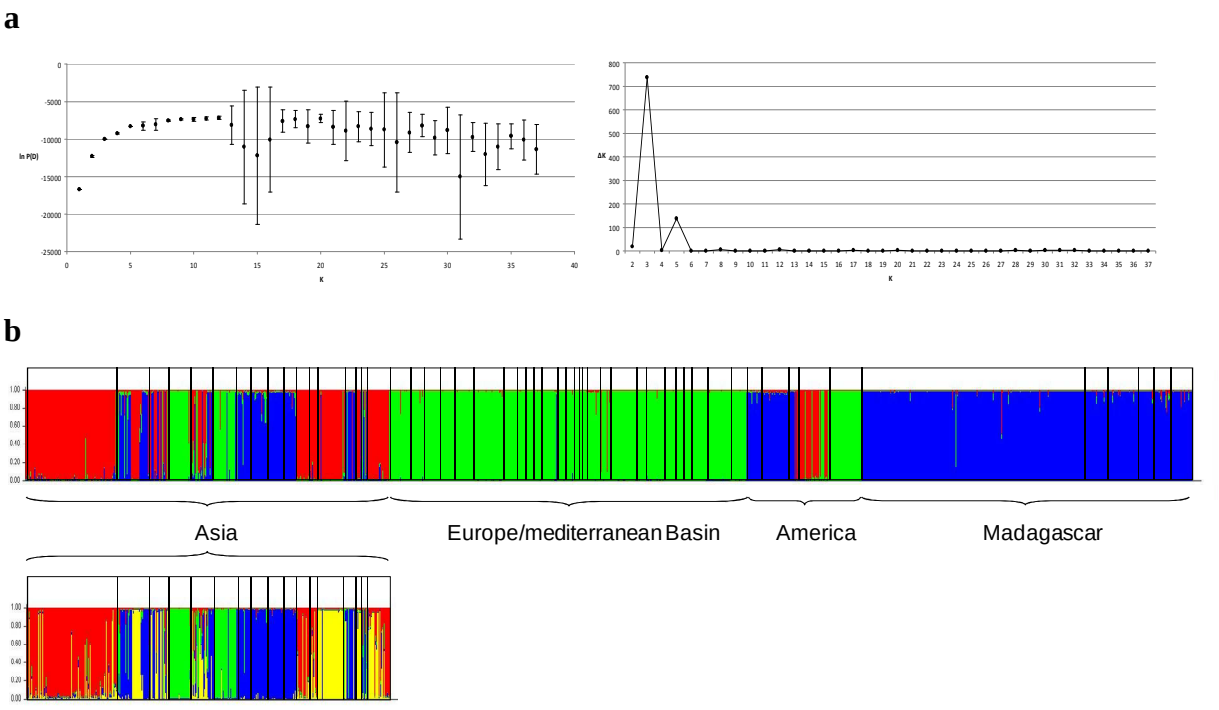
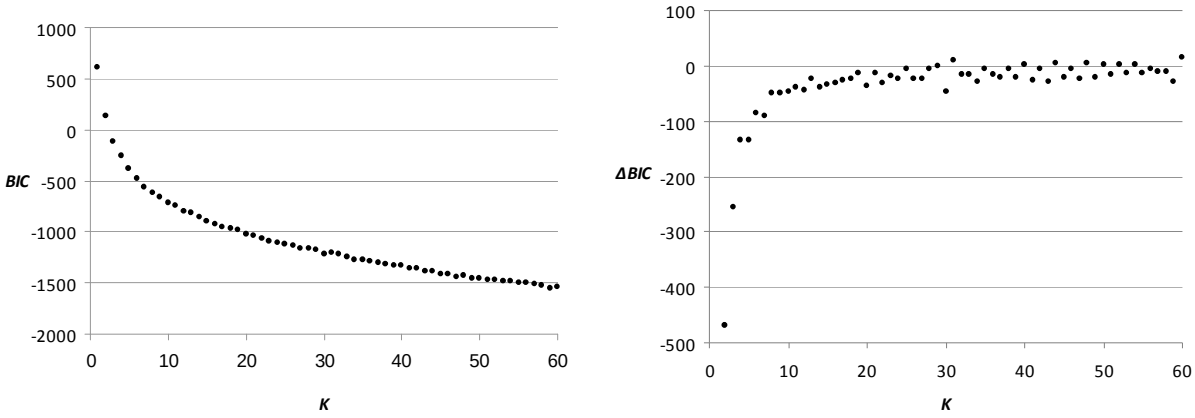


Figure 3.6. Genetic structure of 1372 worldwide individuals of *Magnaporthe oryzae* inferred by the software STRUCTURE, without geographic prior.
a. Determination of the number of clusters (K) using the rate of change in the log probability of data between successive *K* values (Evanno *et al.*, 2005). The rate of change in the log posterior probability of data picks for K=3, indicating that the explanatory value in describing the genetic structure is optimized for this value of K. **b.** Assignments of individuals to each of the three clusters (1-blue, 2-red, 3-green). Each vertical bar represents an individual and the probability of assignment to a cluster is given by the length of the line of the corresponded color. Admixed individuals are represented by two (or more)-coloured lines.

a



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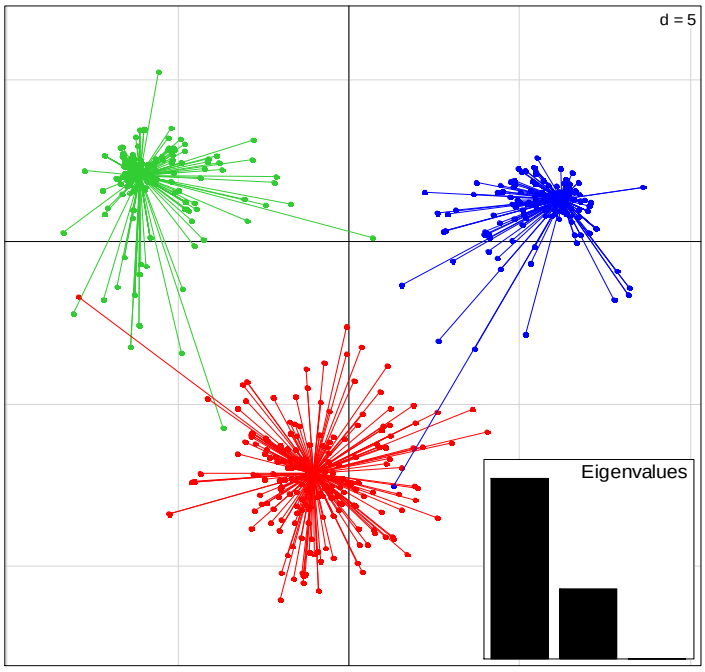

Figure 3.7. DAPC on the 1372 worldwide strains of *M. oryzae*.
a. Bayesian Information Criterion (BIC) as a function of number of clusters *K*. Difference between successive BIC values of *K*. **b.** individuals in the (1,2) plan. The inset gives the eigenvalues of the DAPC.

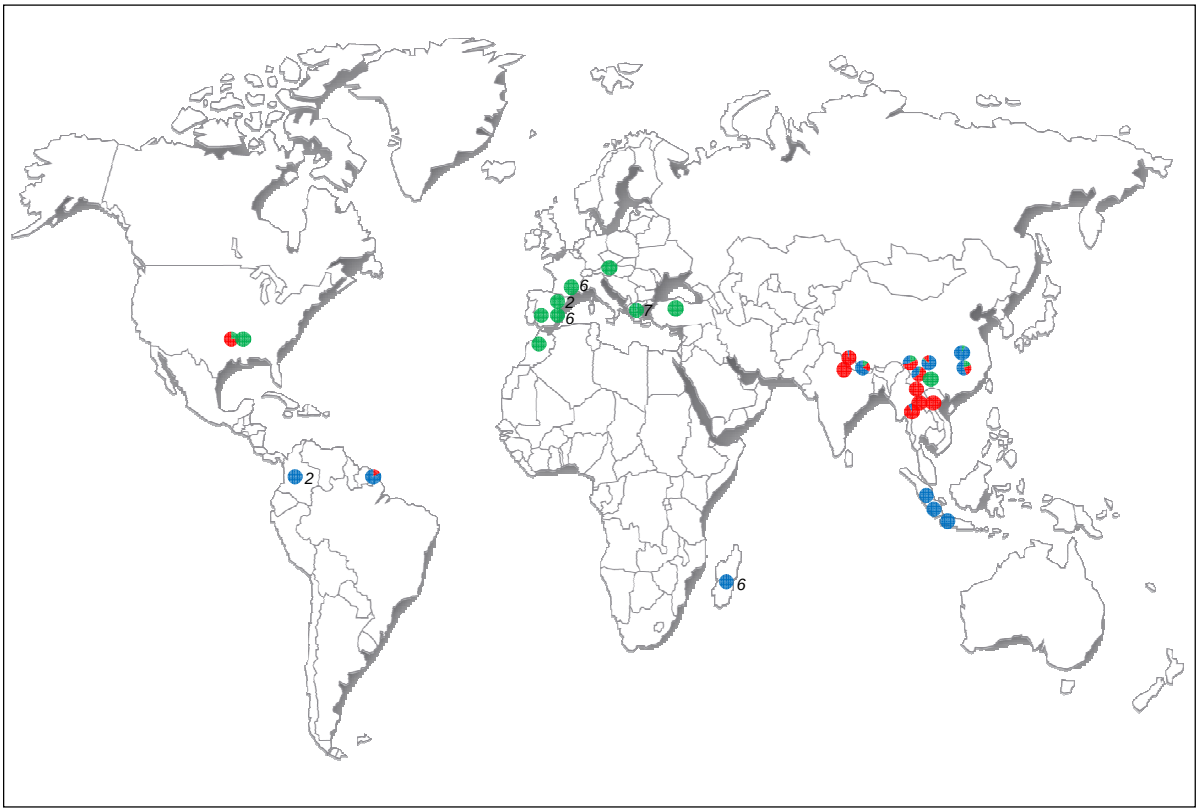

Figure 3.8. Proportion of strains belonging to the three clusters inferred using DAPC in 55 global samples.
The numbers indicate several samples from the same location sharing the same assignment to a cluster. Cluster A: red, cluster B: green and cluster C: blue.

from Europe/Mediterranean Basin belonged to cluster C and all individuals from Madagascar belonged to cluster A. In South America, individuals belonged to cluster A. Individuals from sample USA1 were assigned to two clusters (B and C), which confirmed the presence of two clones in this sample and individuals from sample USA2 belonged to the cluster C.

Interestingly, we found some MLGs that were shared by Asian strains and other strains from Europe, South America and Madagascar. One of the two most observed MLGs in the three Hungarian samples was observed in CH3 and the other was observed in CH4. We also found a MLG in common between the Spanish samples SP2, SP3 and SP4 and CH4. In the Colombian sample CL1, there was one MLG in common with CH2, one MLG in common with CH7, one MLG in common with ID1 and two MLGs in common with ID2. GY and TH shared one common MLG. One genotype observed in MD6 was found in ID2. Finally, in the USA2 sample, one MLG was shared with the Spanish sample SP1 and one MLG was shared with the SP2 sample. This could be explained by migrations between China and Europe, between China, Indonesia and Colombia, between Thailand and French Guyana, between Indonesia and Madagascar and between Europe and North America.

Tree reconstructions between samples using Cavalli-Sforza distance and F_{ST} distance were consistent with clustering analyses (Figure 3.9). European/Mediterranean samples had a single origin and were close to the Chinese samples CH4 and CH6. Malagasy samples had also a single origin and were close to the Chinese sample CH7 and to the three Indonesian samples ID1, ID2 and ID3. South American samples were also close to these four Asian samples. In North American samples, USA1 was close to the Chinese sample CH1, to the Laos samples LA1 and LA2, to the Thai sample TH and to the Nepalese samples NP1 and NP3. As strains from USA1 belong to two clusters corresponding to two clones, this analysis attached the sample to the clone that was the most represented. In the F_{ST} reconstruction, it was attached to European/Mediterranean samples. The USA2 sample was close to European/Mediterranean Basin samples.

We tested the hypothesis of IBD at the European/Mediterranean Basin scale and we found a regression slope significantly different from zero, however it was negative (b=-0.08, P=0.02, figure 3.10). So, samples that were geographically close were more genetically differentiated than samples that were geographically isolated.

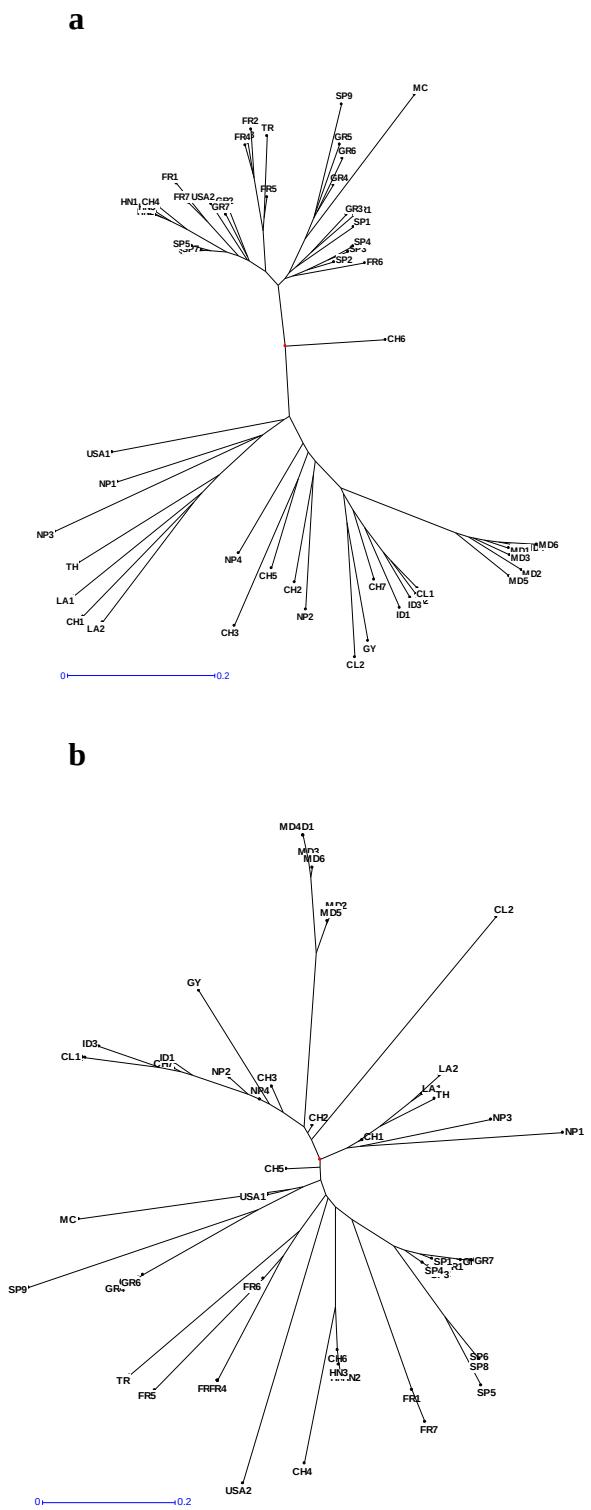

Figure 3.9. Trees constructed on 55 worldwide populations of *M. oryzae* using neighbour-joining method.
a. Cavalli-Sforza distance. **b.** F_{ST} distance.

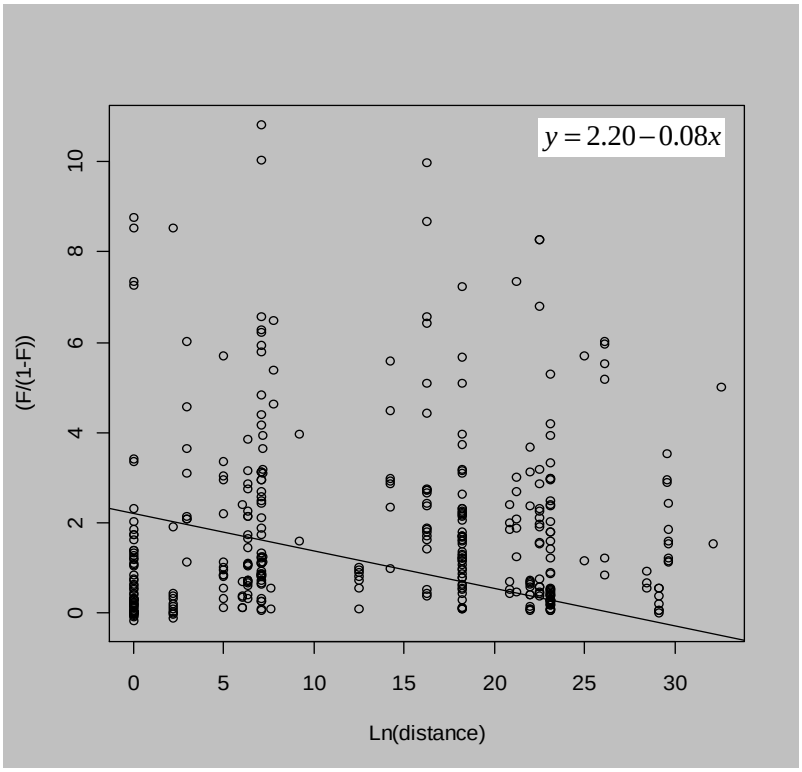

Figure 3.10. Genetic distance (F/(1-F)) as a function of geographic distance (Ln(distance)) between 28 *M. oryzae* populations of Europe/Mediterranean Basin.
The regression equation is given in the inset.

Concordance between genetic structure and biological features.

We studied three factors that could be linked to sample structure at the Asian scale and at the global scale (Table 3.5). At the Asian scale, the assignment to one of the four clusters significantly depends on the type of culture (χ^2 = 72, P<0.05, d.f. 3), the mating type (χ^2 = 330, P<0.05, d.f.= 3) and the female-fertility status (χ^2 = 294, P<0.05, d.f.= 3). Individuals from cluster 2 and 4 were all sampled on irrigated rice. The proportion of female-fertile strains was highly different within the four clusters. Interestingly, the percentage of strains that were female-fertile and belonged to cluster 4 was the highest (30%). Within cluster 4, the proportion of female-fertile strains was three quarters. Within cluster 1, the number of female-fertile and female-sterile strains was balanced. Within clusters 2 and 3, the number of female-fertile strains was low (3 on 69 and 5 on 45 respectively).

At the global scale, the assignment to one of the three clusters significantly depends on the mating type (χ^2 = 441, P<0.05, d.f.= 2) and the female-fertility status (χ^2 = 600, P<0.05, d.f.= 3). Within cluster A, the proportion of mating types and the proportion of female-fertile strains were balanced. In cluster B, there were almost only Mat1 strains (195 on 202) and within cluster C, there were almost only Mat2 strains (329 on 349). Female-fertile strains were rare both within cluster B (3 on 161) and within cluster C (4 on 212).

Discussion

It has been shown that the center of origin of phytopathogenic fungi may be different from the center of diversification and from the center of dispersal (Stukenbrock *et al.* 2008; Gladieux *et al.* 2010). In this study, we provided several evidences that the center of origin of *M. oryzae* on cultivated rice, the center of diversity and the center of dispersal were at a broad scale in the same region: South-East Asia.

Center of diversity

In the present study, the highest genetic diversity was found in Asia, not only at the regional scale (whole South-East Asia) but also at the sample scale, with the exception of Indonesian samples. Allelic diversity was about two times higher, gene diversity was about three times higher, and private alleles diversity was about four times higher in Asian samples

a

cluster	type of culture		mating type		female-fertility	
	irrigated	upland rice	Mat1	Mat2	female-fertile	female-sterile
1	9%	15%	11%	10%	9%	13%
2	**0%**	20%	18%	2%	**1%**	22%
3	7%	15%	7%	17%	2%	14%
4	**0%**	34%	13%	22%	30%	10%

b

cluster	mating type		female-fertility	
	Mat1	Mat2	female-fertile	female-sterile
A	12%	17%	19%	19%
B	25%	**1%**	**1%**	26%
C	3%	42%	**1%**	35%

Table 3.5. Contingency tables of the distribution of individuals in clusters inferred using DAPC with the type of culture, the mating type and female-fertility.
a. At the Asian scale. **b.** At the global scale (information of type of culture was too scarce to be tested). Percentages that are equal to one or less are bolded.

than in samples from Europe/Mediterranean Basin, America and Madagascar. Asian strains formed four genetic clusters. Each of these clusters did not strictly match a single country or region in Asia, but we found a geographic structure in the distribution of the clusters within each sample. This type of structure formed a “mosaic of clusters”, and allowed grouping samples according to the geography. Such a mosaic had already been described for the fungus *Venturia inequalis*, using a similar clustering analysis (Gladieux *et al.* 2008). Some samples were mostly composed of one cluster, and others were composed of two to four cluster. Clusters 1 and 4 showed the highest genetic diversity and especially the highest richness in private alleles. We identified samples that were mostly composed of strains belonging to these two clusters. In an area comprising the Yunnan province of China, Laos and Thailand, the four samples CH1, LA1, LA2 and TH were mostly composed of strains belonging to cluster 4 and a smaller proportion of strains belonging to cluster 1. In Nepal, Western samples (NP1 and NP3) were essentially composed of individuals belonging to cluster 1. Interestingly, these two regions (South Yunnan/Laos/Thailand and Western Nepal) identified here as two centers of important diversity for *M. oryzae*, match the putative centers of domestication of rice, localized in South China and North-East India (Londo *et al.* 2006). Two samples from Eastern part of Yunnan province in China (CH4 and CH6) were mostly composed of strains belonging to cluster 2. One sample from Hunan (CH7), an Eastern province of China, and Indonesian samples were mostly or completely composed of strains belonging to cluster 3. There were common MLGs between Indonesia and CH2 and CH7 samples. So, Indonesian strains might have migrated from the Hunan region of China or from Indonesia to China. As clusters 2 and 3 showed lower genetic diversity compared to clusters 1 and 4, these samples from Eastern Yunnan, Hunan and Indonesia did not represent important centers of diversity compared to South Yunnan/Laos/Thailand and Western Nepal. Finally, the remaining samples were composed of a mosaic of several clusters. These were from Yunnan (CH3 and CH5), Hunan (CH2) and Nepal (NP2). This may be explained either by migrations between the different locations in Asia.

Dispersal towards the rest of the world

The worldwide analysis of population structure allowed hypothesizing that all secondary areas of the pathogens had an Asian origin (Figure 3.5). At the global scale we found three clusters that were consistent with the four clusters found in Asia: the worldwide

clusters B and C corresponded to Asian clusters 2 and 3 respectively and the worldwide clusters A corresponded to Asian clusters 1 and 4 grouped together.

In the European/Mediterranean Basin samples, all individuals but one belonged to a single cluster (cluster B). In Asia, almost all individuals from the Chinese samples CH4 (100%) and CH6 (87%) belonged to this cluster and only 18 individuals from the other Asian samples belonged to this cluster. Moreover, we found common MLGs between Hungarian strains and strains from CH3 and CH4 samples (belonging to cluster B). There were also common MLGs between Spanish strains and CH4 strains. These results allow hypothesizing that strains from Europe and the Mediterranean Basin could originate from the Yunnan province of China where CH3, CH4 and CH6 samples were collected. The hypothesis of a unique South-China origin for the *M. oryzae* European/Mediterranean samples is also supported by the population trees, since all these samples formed a monophyletic clade. This hypothesis of a single Asian origin for all European samples is also reinforced by the fact that only one mating type (Mat1) was found in Europe. The fungus was probably subsequently dispersed from this single entry point throughout Europe. In another study, (Faivre-Rampant *et al.* Annexe 1) we have analyzed an extensive sampling of European *M. oryzae* samples with 11 microsatellites markers and found three genetic groups. The three groups were represented in Western Europe (France, Italy, Spain and Greece) whereas each sample from Eastern countries was composed of only one cluster (Hungary and Turkey). Further analyses at the European scale (Faivre-Rampant *et al.* Annexe 1) suggested that the unique emergence of M. oryzae in Europe was probably followed by important and recurrent long-distance human-mediated migrations throughout the continent. We did not detect IBD in this region.

We found a similar structure indicating single Asian origins for samples in the other parts of the world. South American samples from Colombia and French Guyana belonged to the same cluster (C). CL1 shared MLGs with samples from Western province of China, Hunan (CH2 and CH7) and also with Indonesian samples (ID1 and ID2). CL2 had no MLG in common with any other sample. The most common observed genotype in French Guyana was shared with the Thailand sample. So, *M. oryzae* strains from South America may have different origins depending on the countries: in Colombia, strains might come either directly from Western China or from Indonesia. In French Guyana, strains might have migrated from Thailand. This could be explained by migrations of H'Mongs from Thailand to other parts of the world. In North America, it seemed that the two samples had different origins. The USA1 sample belonged to two clusters (A and B) and did not share any MLG with any other sample.

The USA2 sample belongs to the same cluster as Europe (B) and we found two genotypes in common with Spanish strains. So, North American strains might have migrated from the same genetic pool as Europe in China or from Europe itself.

Malagasy samples had a single origin and belonged to the same cluster as the Chinese sample CH7 and Indonesian samples and one sample shared a common MLG with Indonesia. So, it is likely that Indonesian and Malagasy samples originated from the same genetic pool, or that Malagasy strains migrated from Indonesia.

Our results show single introductions in Europe/Mediterranean Basin, in Madagascar and in South America. They are consistent with the hypothesis suggested by Levy *et al.* (1991) and Zeigler (1998) that *M. oryzae* populations out of Asia recently derived from a limited set of founders and that host selection was important in this zone. Couch *et al.* (2005) also suggested that the contemporary lineages derived from a single sexual lineage because of the clonal structure in introduced areas. On the contrary, in North America, there should have been multiple introductions. All these hypotheses on migration from Asia towards other continents should be compared to historical data and tested using inferential methods such as Approximate Bayesian Computation that allows comparing the likelihood of alternative scenarii and inferring dates on these events (Estoup & Guillemaud 2010). These methods were recently developed for panmictic organisms but will be soon adapted for clonal organisms (Ravigné and Cornuet, pers. com.).

Center of origin

We found that some locations in Asia were centers of diversity and that Asia was the source of migration to other parts of the world. As the center of diversity and the center of dispersal of *M. oryzae* are in the same area, it is not unlikely that Asia is also the center of origin of the species on cultivated rice. It has been suggested that fungal pathogens on cultivated crops that have lost the ability to reproduce sexually may still be able of sexual reproduction near their center of origin (Leslie and Klein 1996). This may be due to the higher genetic diversity in plant cultivars in the center of origin (Stukenbrock and McDonald 2008). We hypothesized that sexual reproduction was possible only in Asia. In Asia in clusters 1 and 4, mating types were equally represented and the proportion of female-fertile strains was high. So, sexual reproduction could have been maintained in strains that belong to these clusters. This was consistent with the observed higher genotypic diversity in these

clusters. The genotypic diversity higher than 60% in some Chinese, Laos, Nepalese and Thai samples reflected the high allelic diversity but also supported the hypothesis of ancient and contemporary sexual reproduction. Moreover, some populations were suspected to have experienced recombination in the Indian Himalayas (Kumar *et al.* 1999).

In addition, the agrosystems were different among the Asian clusters as strains belonging to cluster 1 and 4 were only collected on upland rice. Upland rice varieties are usually more traditional than irrigated varieties. This is consistent with a more ancient origin of strains from South Yunnan/Laos/Thailand and Western Nepal compared to the other samples.

So, we provided evidence that Asia is the most likely center of origin of *M. oryzae* on cultivated rice. We found especially two candidate regions: South Yunnan/Laos/Thailand and Western Nepal. Stukenbrock and McDonald (2008) proposed four main mechanisms involved in the emergence of a pathogen on cultivated plants: host-tracking, host shift or host jump of the pathogen from another host plant, horizontal gene transfer (HGT) between pathogens species and hybridization between pathogens species. Host-tracking consists in the coevolution of the host and the pathogen during domestication. It implies that the host and the pathogen have the same center of origin. This has been suggested for several pathogenic fungi such as *Venturia inequalis* on apple (Gladieux *et al.* 2008), *Mycosphaerella graminicolis* on wheat (Banke & McDonald, 2005), *Ustilago scitaminea* on sugarcane (Raboin *et al.* 2007), *Phytophtora infestans* on potato (Gomez-Alpizar *et al.* 2007) and *Mycosphaerella fijiensis* on banana (Stéphanie Robert, Pers. Comm). Couch *et al.* (2005) suggested that *M. oryzae* on rice originated in China through a host shift from *Setaria* sp. Here we showed that the center of origin of *M. oryzae* pathogenic on cultivated rice was in South East Asia, which is one of the centers of domestication of rice. Both mechanisms (host shift and host tracking) could then have played a role in the emergence of *M. oryzae* on rice. Disentangling between the two processes would require a comparative study of South Asian populations from traditional rice, wild rice *Oryza rufipogon*, and *Setaria* populations coming from the two putative centers of origin.

Conclusion

In this study, we provided a new example of a phytopathogenic fungus for which the center of origin coincides with the center of diversity and the center of dispersal. We also achieved to precise the location of these centers. The two clustering methods gave the same

results. So, even if the hypothesis of panmixia was not verified for STRUCTURE analyses, the method seems robust. This study allowed inferring the origin of worldwide *M. oryzae* rice strains in Asia. It did not allowed inferring the precise history of the dispersal but it allowed making some predictions on introduction events. The worldwide genetic structure observed here may be due to very recent events. Migration routes should now be inferred. The Green Revolution in the sixties has led to a homogenization of crop cultivars around the world (Khush 2001) and may have led to a decrease in pathogens genetic diversity. So, it is important to date the migration events to demonstrate that is has played a role on structuring the populations of *M. oryzae*.

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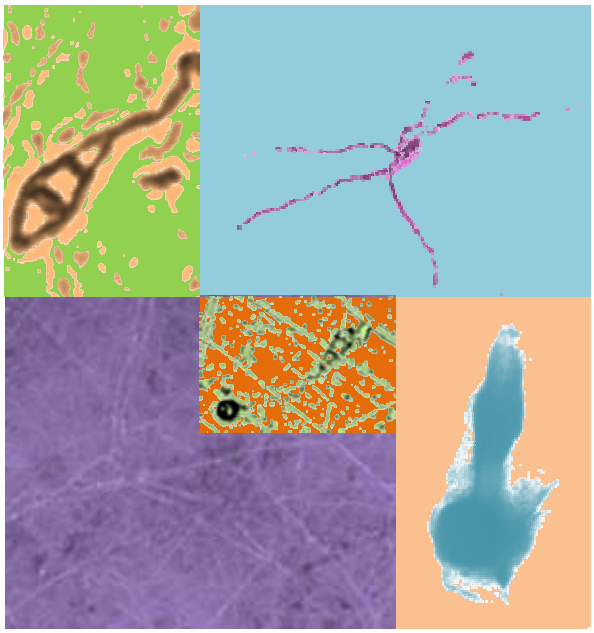
V. Conclusions sur la partie 1.

Dans cette partie, nous avons montré que l'Asie du Sud-Est était le centre d'origine, le centre de diversité et le centre de dispersion de *M. oryzae* sur le riz domestiqué. Nous avons trouvé des centres de diversité en Chine, en Thaïlande, au Laos et au Népal. Nous avons pu rattacher des populations d'Europe et du Bassin Méditerranéen, d'Amérique du Nord et du Sud et de Madagascar à plusieurs centres de dispersion en Chine, en Thaïlande, au Laos, au Népal et en Indonésie. Nous avons aussi montré que la structure génétique à l'échelle mondiale était corrélée aux types sexuels, et que l'Asie était une zone candidate pour l'existence d'événements de reproduction sexuée anciens ou contemporains.

Dans la suite de la thèse, nous avons donc étudié l'évolution du mode de reproduction depuis l'Asie vers le reste du monde.

CHAPITRE 2

Evolution du mode de reproduction chez *Magnaporthe oryzae.*



I. Introduction générale.

1. Etre ou ne pas être sexué : le paradoxe du sexe.

Le mode de transmission du matériel génétique d'une génération à une autre a toujours suscité beaucoup d'intérêt en génétique évolutive (Fisher 1930 ; Haldane 1932 ; Wright 1931 ; Kimura, 1968). Le maintien du sexe, en particulier est une question très étudiée en biologie évolutive (Maynard Smith 1998). En effet, la reproduction sexuée peut être très coûteuse en temps et en énergie pour trouver un partenaire et différencier des organes sexuels (Otto & Lenormand 2002). De plus, un individu ne transmet que la moitié de ses gènes à sa descendance par reproduction sexuée alors qu'il en transmet la totalité par reproduction asexuée (Maynard Smith 1998). La reproduction sexuée peut aussi favoriser la transmission d'éléments parasitaires (Goddard *et al.* 2001). Enfin, un autre effet néfaste de la reproduction sexuée est la cassure de combinaisons alléliques favorables chez un individu adapté à un environnement stable.

Cependant, la reproduction sexuée est très répandue et l'existence d'espèces qui soient strictement clonales est débattue (Welch & Meselson 2000). Kondrashov (1993) a recensé une vingtaine de mécanismes pouvant expliquer les avantages de la reproduction sexuée. Ces avantages sont généralement subdivisés en causes proximales et cause distales. Les causes proximales sont des mécanismes dont le bénéfice est immédiat, comme par exemple la possibilité de réparer l'ADN par la recombinaison. Les causes distales sont des causes évolutives qui permettent d'augmenter la valeur sélective des individus. La reproduction sexuée peut par exemple augmenter la variabilité génétique d'une population, ce qui permet une adaptation plus rapide à un environnement donné par l'effet de la sélection (Otto 2009). Les mécanismes pouvant expliquer l'avantage de la reproduction sexuée ou le coût de la reproduction asexuée sont assez peu illustrés par des exemples empiriques (Meirmans & Neiman 2006). Il existe cependant deux mécanismes très différents mais tous les deux bien renseignés par des exemples (Dybdahl & Lively 1998) et des simulations et modèles théoriques (Howard & Lively 1994 et 1998) : le cliquet de Müller et la dynamique de la Reine Rouge (Howard & Lively 1994 ; Agrawal & Chasnov 2001 ; Siller 2001 ; revue par Rice 2002) (Figure 4.1).

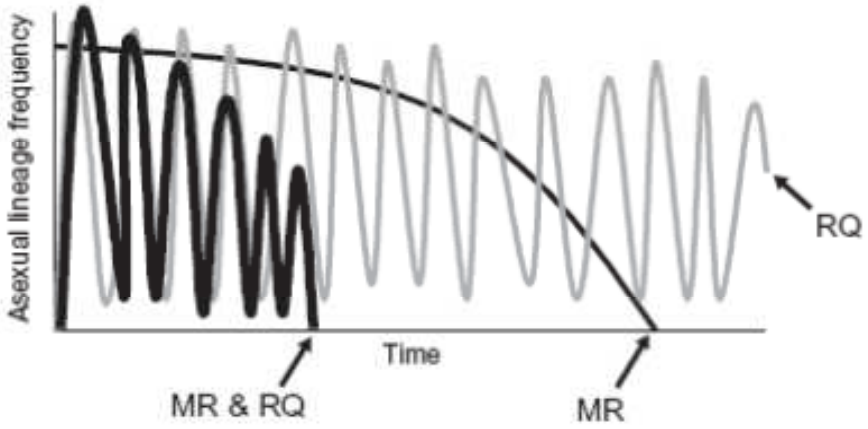


Figure 4.1. Modélisation des effets de l'accumulation de mutations (cliquet de Müller) et de la pression parasitaire (dynamique de la Reine Rouge) sur la fréquence et la longévité des lignées asexuées.
(d'après Meirmans & Neiman, 2006)
L'effet de la pression parasitaire est symbolisé par la ligne grise fine (RQ : *Red Queen*), celui de l'accumulation de mutations est symbolisé par la ligne noire fine (MR : *Müller's Ratchett*), et l'augmentation de la vitesse d'extinction des lignées asexuées par l'interaction entre les deux mécanismes est symbolisé par la ligne noire épaisse (MR & RQ).

Le cliquet de Müller est un terme utilisé pour décrire l'accumulation de mutations délétères sous régime de reproduction asexué. En effet, dans des populations non sexuées et de taille finie, les mutations délétères s'accumulent sur des loci qui ne sont pas sous sélection et le génome entier est transmis d'une génération à l'autre (Müller, 1964). A l'inverse, il a été montré que la reproduction sexuée pouvait ralentir ce processus d'accumulation de mutations délétères (Bruggeman *et al.* 2003).

La métaphore de la Reine Rouge, décrivant la co-évolution entre hôte et agent pathogène, a été reprise par Bell (1982) pour expliquer les interactions entre hôtes et agents pathogènes, et comment la pression parasitaire peut jouer comme force permettant aux hôtes de maintenir un mode de reproduction sexué. En effet, si dans une population d'hôtes se reproduisant sexuellement, un clone asexué envahit la population, alors les agents pathogènes qui seront le plus sélectionnés sont ceux pouvant infecter le génotype clonal. Ainsi, le génotype clonal ne remplacera pas les génotypes sexués (Lively 2010 ; Jokela *et al.* 2009).

Il n'existe cependant aucune étude se plaçant du point de vue de l'agent pathogène, c'est-à-dire sur l'influence de l'hôte sur l'évolution du sexe chez l'agent pathogène. Si l'on se place dans un milieu domestiqué, la reproduction de l'hôte est contrôlée et la diversité génétique totale dans la population (le champ) est la plupart du temps nulle. Nous pouvons donc faire l'hypothèse qu'un agent pathogène adapté au génotype cultivé et se reproduisant de manière clonale a une probabilité élevée d'envahir la population d'hôtes, contre-sélectionnant ainsi les agents pathogènes recombinants. En revanche, dans un mode de culture plus traditionnel avec cohabitation de différents génotypes, voire de cultivars, un agent pathogène recombinant sera toujours sélectionné.

La reproduction sexuée modifie les associations entre chromosomes par la ségrégation chez les organismes polyploïdes et les associations entre allèles par la recombinaison chez tous les organismes. La recombinaison incluse des processus méiotiques (ségrégation suivie de l'échange de fragments d'ADN entre chromosomes homologues à partir d'origines parentales uniques ou différentes) mais aussi des processus non méiotiques tels que la transformation chez les bactéries, le saut de matrice chez les virus (Halkett *et al.* 2005), et la parasexualité chez les champignons (Zeigler *et al.* 1997). Par opposition, la reproduction clonale comprend toutes les formes de reproduction pour lesquelles un individu est produit à partir d'un seul parent sans qu'il y ait de recombinaison génétique (Halkett *et al.* 2005). La

réorganisation génétique, dans ce cas, est due uniquement à des mutations et des modifications chromosomiques.

2. Estimation de la recombinaison

La recombinaison affecte les patrons d'associations entre allèles que l'on peut observer dans les populations. Ainsi, les méthodes d'estimation se basent sur cette propriété et un certain nombre de prédictions peuvent être faites si la recombinaison a lieu : la diversité génotypique devrait augmenter, tous les recombinants génotypiques devraient être observés pour la plupart des paires de loci polymorphes et les différentes régions du génome devraient avoir des histoires de descendance différentes (Milgroom 1996).

Les indices de diversité génotypique sont couramment utilisés pour estimer la recombinaison (Halkett *et al.* 2005). Cette diversité peut être estimée simplement par la proportion de genotypes multilocus (MLG) dans un échantillon. D'autres indices comme celui de Stoddart & Taylor (1988) permettent d'estimer non seulement la richesse en MLG mais aussi l'uniformité de la distribution des différents MLG dans un échantillon (Grünwald *et al.* 2003).

Le *Four Gamete Test* (Hudson & Kaplan 1985), permet quant à lui de détecter des évènements de recombinaison entre deux loci. Il se base sur l'hypothèse que si entre deux loci bi-alléliques les quatre combinaisons alléliques possibles sont observées dans un échantillon, alors il y a eu recombinaison entre les deux loci. Les deux loci sont alors qualifiés d'incompatibles. Ce test existe pour les loci présentant plus de deux allèles (Estabrook & Landrum 1975).

Les mutations apparaissent indépendamment sur des loci uniques alors que les effets du sexe s'étendent au génome. Ainsi, une nouvelle mutation se produit sur un unique individu et est en complète association avec le polymorphisme porté par le chromosome. La reproduction clonale affecte la dynamique des gènes en empêchant le remaniement des allèles entre les loci (Halkett *et al.* 2005). En revanche, les associations entre loci sont cassées par le processus de recombinaison ce qui fait qu'en théorie le degré d'association (LD) entre allèles

dans un échantillon de chromosomes est théoriquement simplement fonction de l'âge de la mutation et du taux de recombinaison.

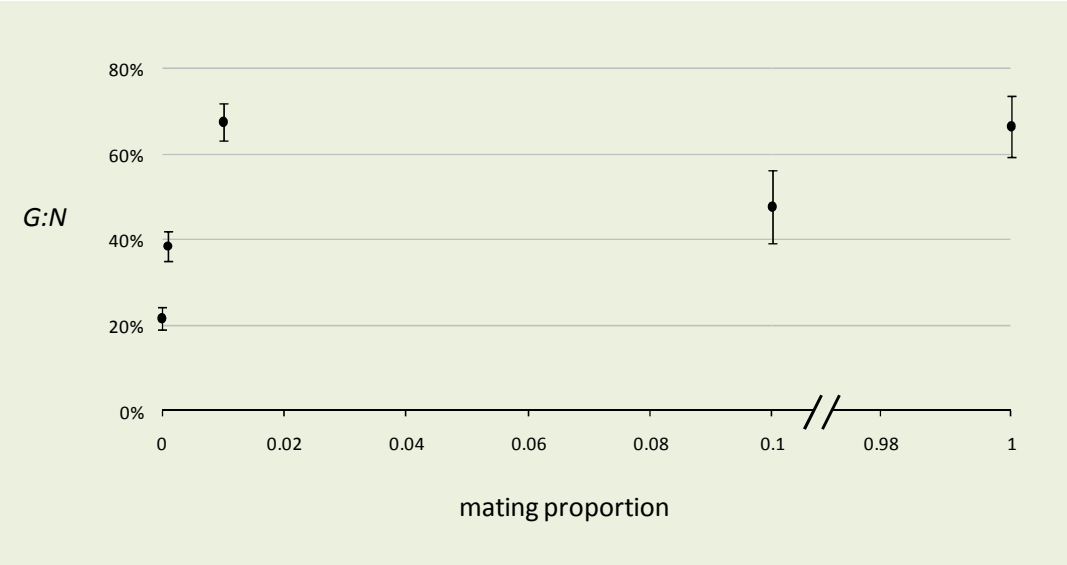
L'indice d'association I_A est un estimateur de la recombinaison puisqu'il permet la mesure du déséquilibre de liaison multilocus (Agapow & Burt 2001). Cette statistique est basée sur la variance des distances deux à deux entre individus (c'est-à-dire le nombre de loci auxquels ils diffèrent). Elle permet de tester dans quelle mesure des individus qui sont identiques pour un locus sont plus susceptible d'être identiques pour d'autres loci que par le simple fait du hasard. Elle est calculée dans un échantillon selon la formule :

$$I_A = (V_o / V_e) - 1$$

V_o représente la variance de la distance observée entre chaque paire d'individus (nombre de loci pour lesquels ils présentent des allèles différents) et V_e représente la variance de la distance entre chaque paire d'individus attendue sous hypothèse de panmixie. La valeur minimale est de 0 s'il y a panmixie, et augmente avec le taux de clonalité. L'indice $\overline{r_D}$ permet de corriger la statistique du I_A pour le nombre de loci étudiés. Il est compris entre 0 (panmixie) et 1 (clonalité stricte). Sous régime de clonalité, les coefficients de déséquilibre de liaison devraient être proches de leur maximum théorique, et indépendants de la distance physique entre les gènes (Maynard Smith *et al.* 1993). Une question importante est de savoir à quelle fréquence les recombinaisons doivent se faire pour qu'une population apparaisse panmictique (I_A=0). De Meeûs & Balloux (2004), par une approche de simulations de jeux de données microsatellites diploïdes, ont montré que les indices d'association multilocus ne variaient pas linéairement avec le taux de recombinaison et dépendaient de forces évolutives indépendantes du régime de reproduction (en particulier la taille des populations et la migration entre populations). Nous avons obtenu des résultats similaires sur des simulations sur jeux de données microsatellites, en utilisant le logiciel QuantiNemo (version 1.0.2 adaptée pour les haploïdes) (Figure 4.2). De plus, les évènements historiques de recombinaison peuvent laisser une signature dans les données de génétique des populations (Stumpf & Mc Vean 2003) et être interprétés à tort comme des évènements de recombinaison contemporains.

Enfin, certaines méthodes se basent sur la coalescence pour construire un arbre ancestral de recombinaison et estimer un taux de recombinaison par génération dans un échantillon (Hudson 1987 ; Hey et Wakeley 1997 ; Fearnhead et Donnelly 2001) mais nécessitent un grand nombre de marqueurs génétique neutres.

a



b

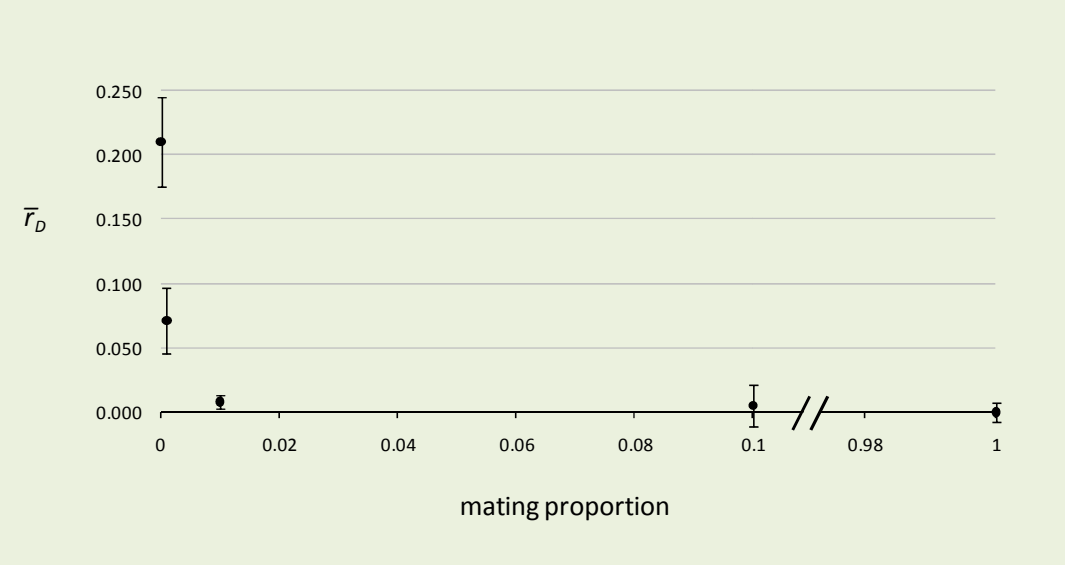


Figure 4.2. Valeurs de la proportion de génotypes multilocus (a) et du déséquilibre de liaison multilocus (b) en fonction de la proportion de recombinants dans une population. Les deux indices ont été calculés sur des jeux de données haploïdes de 17 marqueurs microsatellites simulés à l'aide du logiciel Quantinemo (version 1.0.2 modifiée pour les haploïdes). Cinq conditions ont été testées sur la proportion de recombinants : 0.0001, 0.001, 0.01, 0.1, et 1. Pour chaque condition, 9 populations de taille initiale 1000 ont été simulées avec un taux de migration de 0.5 suivant en modèle en îles (la moitié des individus d'une population est redistribuée dans les 8 autres populations à chaque génération). Le nombre initial d'allèles par locus a été fixé à 25 et le taux de mutation à 10^{-5} mutations par individu et par génération. Deux réplicats ont été simulés pour chaque valeur de proportion de recombinants, permettant d'obtenir 18 populations par condition. Après 60000 générations, un échantillonnage aléatoire de 30 individus a été réalisé dans chaque population. Les valeurs d'indices présentées sur ces graphiques représentent la moyenne sur 18 populations et les barres d'erreurs les intervalles de confiance corrigés par le nombre de réplicats.

Il existe un certain nombre d'organismes haploïdes, tels que les bactéries et les champignons qui se propagent par le mode asexué exclusif ou couplé avec une reproduction sexuée toutefois moins importante. Dans ce cas, il est difficile de déterminer la part de recombinaison sexuée participant à cette structure. En effet, si une population connait une forte reproduction clonale après un évènement de reproduction sexuée, les traces de recombinaison sont plus difficiles à estimer (Maynard Smith *et al.* 1993). Inversement, dans les populations principalement clonales, les mutations peuvent contribuer au taux d'une sexualité apparente (Thompson 2007). Ainsi, afin d'estimer la recombinaison de manière correcte, il est important d'utiliser simultanément différentes approches.

3. La reproduction sexuée chez les champignons phytopathogènes.

Il est important d'estimer la recombinaison chez les agents pathogènes puisque les évènements occasionnels de recombinaison sexuelle chez les organismes clonaux ont des conséquences en termes de dynamique des populations et de trajectoire évolutive (Halkett *et al.* 2005). La recombinaison joue en effet un rôle important dans l'évolution de nouveaux pathotypes capables de s'adapter à des variétés résistantes et dans l'évolution de la résistance aux fongicides (Milgroom 1996). A cause de l'absence complète de recombinaison chez les organismes strictement clonaux, toute mutation dominante fortement délétère conduit une lignée à l'extinction. Les nouvelles mutations bénéfiques réduisent aussi les tailles de populations efficaces des clones puisque les lignées possédant la nouvelle mutation bénéfique vont remplacer les autres lignées (Balloux *et al.* 2003).

Dans le règne des champignons, des espèces très étroitement apparentées exhibent souvent des systèmes de reproduction divers, allant de très sexué à complètement asexué, et un nombre de gamètes très variables (Billiard *et al.* 2011). Chez ces organismes, la reproduction sexuée implique la sécrétion de phéromones induisant la fusion de deux cellules, et d'autres facteurs chimiques, par exemple des molécules lipidiques qui jouent un rôle dans les changements morphogénétiques liés à la reproduction sexuée (Coppin *et al.* 1997). Chez les champignons hétérothalliques, cette fusion n'est possible qu'entre deux souches de types sexuels (*mating types*) opposés. Alors que chez les champignons homothalliques, elle peut aussi se produire entre deux cellules d'une même souche (Heitman 2010). Les types sexuels

sont déterminés par des gènes localisés dans des régions dites *MAT*. Chez les ascomycètes filamenteux, ces régions peuvent contenir jusqu'à trois gènes (Dyer 2008). C'est le cas chez le champignon *Podospora anserina* dont les gènes *MAT* jouent un rôle dans la formation des organes femelles après la fertilisation (Debuchy *et al.* 1993). Des gènes de méiose communs à la majorité des Eucaryotes sexués ont été identifiés chez les champignons (Schurko & Logsdon 2008).

4. La reproduction chez *Magnaporthe oryzae*.

M. oryzae est un champignon hétérothallique. Il existe deux types sexuels, Mat1 et Mat2 essentiellement contrôlés par deux gènes idiomorphes situés sur un même locus (Figure 4.3). La reproduction sexuée *in vitro* nécessite la mise en contact des mycéliums de deux souches de types sexuels opposés (Figure 4.4, Annexe 2). Au moins une des deux souches doit être femelle-fertile, c'est-à-dire capable de former les périthèces, organes femelles dans lesquels sont produites les spores sexuées –les ascospores- regroupées par huit dans un asque. Les ascospores germent ensuite pour donner un nouveau mycélium, produit de la reproduction sexuée entre les deux souches initiales. Les mécanismes exacts de la reproduction sont encore peu connus. Kato *et al.* (1994) ont mis en évidence la production de microconidies chez *M. oryzae.* Ces résultats ont été confirmés par Chuma *et al.* (2009) et Zhou *et al.* (2011). Le rôle des microconidies dans la fertilité mâle lors de la reproduction sexuée, n'est pas démontré chez *M. oryzae* contrairement à *Podospora anserina*, *Botrytis cinerea* et *Neurospora crassa*.

Chez les champignons, en particulier chez les ascomycètes, il peut y avoir un cycle parasexuel qui implique la fusion de deux noyaux haploïdes et une recombinaison sans méiose (Schurko & Logsdon 2008). Plusieurs auteurs ont suggéré des mécanismes de parasexualité en plus de la reproduction sexuée chez *M. oryzae* (Genovesi *et al.* 1976 ; Zeigler *et al.* 1997 ; Noguchi *et al.* 2006). Ils pourraient influencer l'estimation de la reproduction sexuée puisqu'ils génèrent aussi de la recombinaison. Toutefois, l'importance de ce phénomène dans les populations naturelles n'est pas connue.

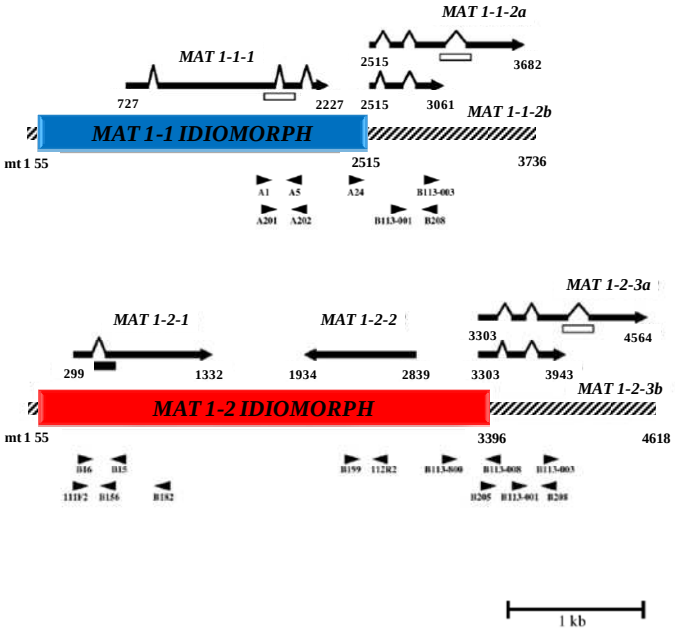


Figure 4.3. Structure du locus *MAT* déterminant les types sexuels chez *M. oryzae*. (d'après Kanamori *et al.*, 2007)

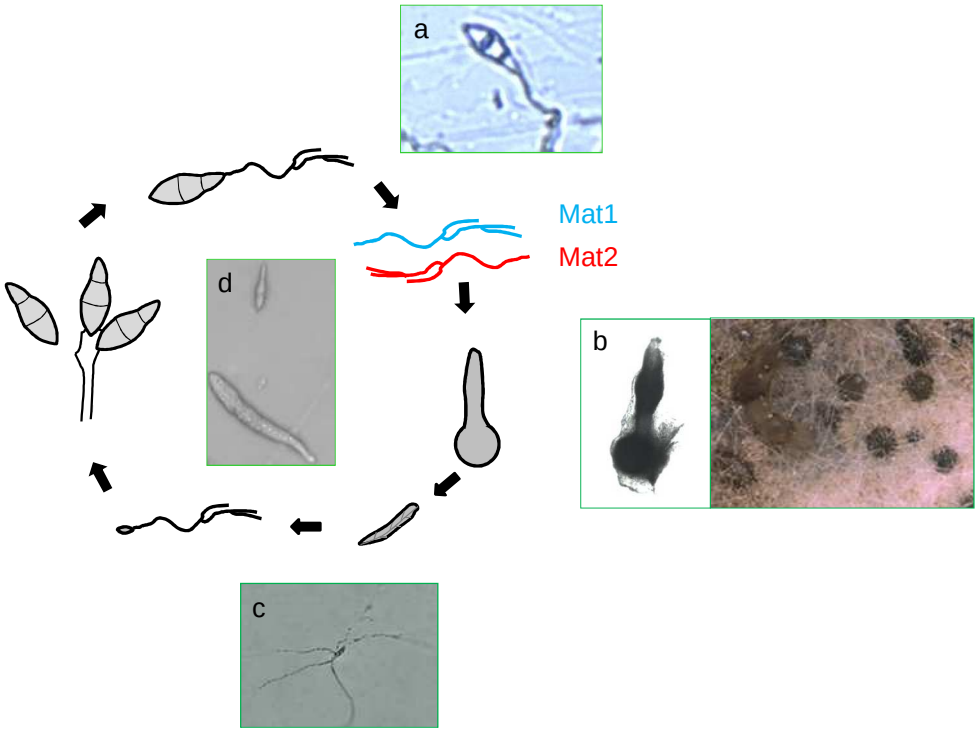


Figure 4.4. Cycle sexuée de *M. oryzae*. Photos. a : conidie germée ; b : périthèce isolé (à gauche) et sur boite de Pétri (à droite) ; c : asque germé ; d : comparaison d'une conidie (en haut et d'un asque (en bas).

5. Reproduction sexuée dans le centre d'origine et perte de la reproduction sexuée dans les aires introduites.

Il a été suggéré que les champignons filamenteux considérés comme ayant complètement perdu la capacité de reproduction sexuée, en soient encore capables près du centre d'origine de ces espèces ou dans des environnements hétérogènes ou variables (Leslie & Klein 1996). Dans le chapitre 1, nous avons confirmé que le centre d'origine de *M. oryzae* sur le riz était en Asie du Sud-Est. C'est aussi la seule région du monde où des souches femelle-fertiles ont été trouvées, à l'exception de deux souches provenant de Guyane française mais ayant probablement été importées accidentellement depuis l'Asie par les H'mongs dans les années 1970. Les deux types sexuels ont été identifiés en proportions équilibrées en Asie (Nottéghem & Silué 1992 ; Zeng *et al.* 2009). Dans le reste du monde, il existe généralement un type sexuel qui est plus représenté que l'autre et certaines régions comme l'Europe ne comprennent qu'un seul type sexuel (Nottéghem & Silué 1992). L'Asie est aussi une région où des souches appartenant à des types sexuels différents peuvent être trouvées dans la même population. C'est donc la région candidate idéale pour la détection de la reproduction sexuée. La recombinaison a été suggérée dans une population d'Inde (Kumar *et al.* 2004) et en Thaïlande (Mekwatanakarn *et al.* 1999).

Dans un premier temps, nous avons cherché à tester l'hypothèse selon laquelle la reproduction sexuée de *M. oryzae* aurait lieu en Asie. Notre démarche a donc tout d'abord été d'étudier la distribution et le ratio des deux type sexuels et des souches femelle-fertiles au niveau populationnel à l'échelle mondiale afin de cibler les zones où la reproduction sexuée peut effectivement avoir lieu. De plus, nous avons cherché à détecter la recombinaison dans chacune de ces populations. Ces résultats sont présentés dans la partie II de ce chapitre sous la forme d'un article soumis (Article 2) et d'analyses supplémentaires.

Dans un deuxième temps, nous avons testé l'hypothèse de perte de la capacité de reproduction sexuée par la perte de fertilité femelle en réalisant une étude d'évolution expérimentale. Ces résultats sont présentés dans la partie III de ce chapitre sous forme d'un article soumis (Article 3).

II. La reproduction sexuée existe-t-elle chez *M. oryzae* ?

1. Article 2. Sex at the origin: Discovery in Asia of a sexually reproducing population of *Magnaporthe oryzae*—the causal agent of rice blast disease.

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CHAPITRE 2

Sex at the origin: an Asian population of the rice blast fungus *Magnaporthe oryzae* reproduces sexually

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Running title: Sexual reproduction in *Magnaporthe oryzae*

Abstract

Knowledge about the occurrence of sexual recombination within natural populations is essential for their control. However, sexual reproduction may be cryptic or facultative in fungi and therefore difficult to detect. *Magnaporthe oryzae,* which causes blast, the most damaging fungal disease of rice, is thought to originate from South-East Asia. It reproduces asexually in most rice-growing regions. Sexual reproduction has been suspected in limited areas of South-East Asia, but has never been demonstrated in contemporary populations. We tested the hypothesis that this species reproduces sexually in South-East Asia. We characterized several *M. oryzae* populations worldwide both biologically and genetically, to identify candidate populations for sexual reproduction. The sexual cycle of *M. oryzae* requires two strains of opposite mating types, at least one of which is female-fertile strain, to come into contact. In one Chinese population, the two mating types were found to be present at similar frequencies and almost all strains were female-fertile. Compatible strains from this population completed the sexual cycle *in vitro* and produced viable progenies. Genotypic richness and linkage disequilibrium data also supported the existence of sexual reproduction in this population. We resampled this population the following year, and the data obtained confirmed the presence of all the biological and genetic characteristics of sexual reproduction. In particular, a considerable genetic reshuffling of alleles was observed between the two years. Computer simulations confirmed that the observed genetic characteristics were unlikely to have arisen in the absence of recombination. We therefore concluded that a contemporary population of *M. oryzae,* pathogenic on rice, reproduces sexually *in natura* in South-East Asia. Our findings provide evidence for the loss of sexual reproduction by a fungal plant pathogen outside its centre of origin.

Introduction

The ability of organisms to adapt to environmental conditions is tightly linked to their mode of reproduction. Sexual reproduction shuffles existing genetic material, generating selectable genetic variation (Otto & Lenormand 2002; Otto 2009). Segregation and recombination then eliminate deleterious mutations and fix beneficial mutations (Fisher 1930; Muller 1932, 1964), increasing the efficiency of natural selection. This process is particularly important for species confronted with changing environments, such as pathogens encountering new hosts or new agricultural practices, as demonstrated both theoretically (Otto & Michalakis 1998) and experimentally (Goddard *et al.* 2005; Grimberg & Zeyl. 2005; Perlstein *et al.* 2007; Zhan *et al.* 2007). Conversely, an absence of recombination ensures that beneficial associations between loci are maintained. This makes it possible to retain local adaptations, once they have occurred (Fisher 1930; Maynard Smith 1978; Feldman *et al.* 1980; Charlesworth & Barton 1996). Many fungal pathogens get the best of both worlds by alternating recombination and vegetative reproduction. This strategy enables them to adapt to changes in agricultural practice and threatens the durability of control measures (McDonald & Linde 2002).

Fungal plant pathogens can easily overcome the resistance of new resistant cultivars: they escape plant surveillance systems, which are often determined by single resistance genes, by modifying a single so-called “avirulence” gene (Jones & Dangl 2006). In these gene-for-gene interactions, recombination facilitates the adaptation of the pathogen, by combining favorable mutations at multiple avirulence loci. Many of the strategies widely used in breeding for resistance, such as the use of combinations of several resistance genes (pyramiding), are unlikely to be effective in populations displaying sexual reproduction, because a multivirulent strain may easily arise through recombination followed by selection. Thus, deciphering the mode of reproduction of pathogens is an important step towards the definition of durable control strategies (Zeyl 2009).

It is difficult to detect sexual reproduction in populations in which sex is cryptic or facultative. Sexual organs may be hard to observe, as sexual reproduction may be restricted to limited spatial areas or periods of time. High genotypic diversities and low levels of linkage disequilibrium measured with molecular markers can be used to search for genetic signatures of recombination (Balloux *et al.* 2003; De Meeûs & Balloux 2004; Halkett *et al.* 2005; Arnaud-Haond *et al.* 2007). This requires that appropriate population samples are available, i.e. substantial samples from recombinant populations. Recent studies based on biological or

molecular approaches and including well studied human pathogens have suggested that several fungi long thought to be exclusively asexual actually reproduce sexually (Taylor *et al.* 1999), in either controlled conditions (Hull *et al.* 2000; Horn *et al.* 2009; O'Gorman *et al.* 2009), or natural populations (Burt *et al.* 1996; Matute *et al.* 2006). Other studies have reported the presence of mating type genes in the genomes of strictly clonal species and in species for which no sexual cycles has been described, probably reflecting a recent loss of the ability to reproduce sexually (e.g. Wong *et al.* 2003; Galagan *et al.* 2005; Fisher 2007; Hoff *et al.* 2008; López-Villavicencio *et al.* 2010). However, the occurrence of sexual reproduction in contemporary populations *in natura* remains difficult to prove unequivocally.

Magnaporthe oryzae is the fungus responsible for blast disease in rice and other grasses; it is considered a model phytopathogenic fungus species (Wilson & Talbot 2009). The genetic structure of *M. oryzae* populations pathogenic on rice suggests that reproduction is clonal in most rice-growing areas (Colombia, USA, Europe, Korea, Japan: Zeigler 1998 for a review; Morocco: El Guilli *et al.* 2005; Madagascar: Andriantsimialona and Tharreau 2008). Moreover, despite careful investigation, perithecia (the organs in which meiosis takes place) have never been reported in the field (Zeigler 1998). However, sexual reproduction has been shown to occur *in vitro* between strains sampled *in natura* (Silué & Nottéghem 1990; Hayashi *et al.* 1997). As in other heterothallic Ascomycetes, the sexual cycle of this fungus requires two strains of opposite mating types (MAT1 / MAT2), at least one of which must be female-fertile (able to produce perithecia). Female fertility is thus a key biological characteristic underlying the mode of reproduction of *M. oryzae***.** In this species, female-fertile strains have been collected from rice only very rarely, and only in the Yunnan province of China, Northern Thailand, and Northern India (Zeigler 1998; Kumar *et al.* 1999; Mekwatanakarn *et al.* 1999). Southern China and Northern India are the two domestication and diversification centres of Asian cultivated rice, *Oryza sativa* (Londo *et al.* 2006). Genetic diversity analyses have identified the Himalayan foothills as the most likely centre of origin of the *M. oryzae* populations pathogenic on rice (Zeigler 1998; Tharreau *et al.* 2009). Based on these findings, it has been suggested that this fungus may reproduce sexually in South Asia, close to the Himalayan foothills (Zeigler 1998; Tharreau *et al.* 2009). Worldwide migrations would have been accompanied by a decrease in the diversity of this species (Zeigler 1998; Tharreau *et al.* 2009) and a loss of its ability to reproduce sexually. Bottlenecks accompanying migrations and/or selective effects due to differences in fitness between strains of the two mating types (Soubabère *et al.* 2000; Couch *et al.* 2005) may have led to the fixation of a single mating

type in invaded areas, imposing an obligate asexual mode of reproduction on pathogen populations outside the centre of origin. The testing of these hypotheses requires an initial demonstration that *M. oryzae* reproduces sexually in its putative centre of origin. Previous studies have suggested that *M. oryzae* may reproduce sexually in this region, based on the observation of both mating types and of female-fertile strains in restricted areas of Asia (Zeigler 1998). Both these criteria are essential for sexual reproduction to occur, but they are not sufficient to prove that sexual reproduction is still occurring in this area. Only Kumar *et al.* (1999) have provided genetic evidence of sexual reproduction in a *M. oryzae* population pathogenic on rice. Kumar *et al.* (1999) detected female-fertile strains and both mating types in two Indian populations. As expected, genotyping detected identical multilocus genotypes (MLGs) due to clonal reproduction. However, no significant linkage disequilibrium (LD) was observed after clone correction, suggesting that some recombination had occurred. Unfortunately, significant LD was detected the following year, and the authors concluded that "It cannot be inferred from the data whether the recombination events producing the present Himalayan populations are ongoing" (Kumar *et al.* 1999). It therefore remains to be determined whether sexual reproduction is currently occurring in rice-specific *M. oryzae* populations.

In this study, we combined biological and population genetics approaches to determine whether sexual reproduction was occurring in certain Asian populations. We also contrasted the features of these populations with those of supposedly clonal populations from other continents. We first searched for biological signatures of sexual reproduction in nine populations from around the world, by determining the relative proportions of the two mating types and the frequency of female-fertile strains. We then searched for molecular signatures of recombination, using microsatellite markers and determined whether the genetic characteristics of the nine populations studied could have resulted exclusively from clonal reproduction, by comparing the observed data with simulations for exclusively asexual populations.

Materials and Methods

Strain collection, isolation and storage

We used the CIRAD *Magnaporthe oryzae* collection to analyze mating type and female fertility. This collection is comprised of strains isolated from cultivated rice, in 55

Origin	Population	*N*	*G*	*CG*	*CG:N*	*STG:N*	N_a	H_e	$\overline{r_D}$	*Comp*
Thailand	TH	27	20	18	67%	55%	4.1	0.47	0.14	58.1%
China	CH1-2008	24	21	18	75%	80%	5.2	0.63	0.16	27.2%
China	CH1-2009	83	76	63	76%	86%	7.1	0.64	0.07	0.7%
China	CH2	38	32	21	55%	64%	3.8	0.50	0.21	47.1%
China	CH3	23	14	14	61%	43%	4.1	0.50	0.25	83.1%
China	CH4	25	5	4	16%	13%	1.5	0.08	0.62	100%
Colombia	CL	31	9	2	6%	11%	1.5	0.06	NT	100%
France	FR	23	12	4	17%	22%	1.6	0.09	0.30	100%
USA	USA	37	20	6	16%	31%	3.2	0.56	0.57	97.8%
Madagascar	MD	95	18	10	11%	3%	2.1	0.07	0.31	97.1%

Table 5.1 Genetic diversity and multilocus linkage disequilibrium in the studied populations. *N*: sample size (number of genotyped strains). *G*: number of multilocus genotypes. *CG*: number of clonal groups (calculated with e-Burst, see methods). *CG:N*: clonal richness. *STG*: Stoddart and Taylor's genotypic diversity index standardized by dividing by sample size. N_a: mean number of alleles per locus. H_e: gene diversity. $\overline{r_D}$: multilocus association index, calculated from data corrected for mutation (see methods). *Comp*: Proportion of compatible pairs of loci. $\overline{r_D}$ was significantly different from 0 in all populations (as assessed with MULTILOCUS 1.3 after 1000 randomizations).

countries, over the last 40 years. For detailed population studies, strains were collected, between 1994 and 2009, from cultivated rice in nine populations (the term “population” is used to refer to all the fungal strains obtained from the same field, on the same date, for the same rice variety) from five countries: China, USA, Madagascar, France, Colombia (Table 5.1). One population (CH1) was sampled in two consecutive years (2008 and 2009). The TH population was isolated from a barley seed lot from Thailand. Barley has been shown to be susceptible to several *M. oryzae* genetic subgroups specializing on different host plants, including rice. The TH population was shown to have all the genetic and pathogenicity characteristics of populations of *M. oryzae* attacking rice (D. Tharreau, unpublished), and could therefore be considered a “rice” population. These populations represented a total of 456 strains (23 to 108 strains per population), which were considered representative of all continents and of the genetic diversity already observed in a worldwide collection of more than 2000 rice-infecting strains (Tharreau *et al.* 2009). Fungal strains were isolated from infected plant material placed in humid chamber at 21°C for 1-2 days, and genetically pure fungal strains were obtained by monospore isolation. Fungal strains were grown on rice flour medium, as previously described (Silué & Nottéghem 1990), and were stored on filter paper at -20°C, as described by Valent *et al.* (1986).

Crosses for mating type and female-fertility determination

Mating type and female fertility were determined as previously described (Nottéghem & Silué 1992). Each strain to be tested was cocultured on rice-flour medium with two reference female-fertile strains of each mating type. Reference strains are strains of known mating type that produce and induce the production of perithecia when cocultured with strains of the opposite mating type. We assessed perithecium production along the line of contact between the strain tested and the reference strain with which it was cocultured, after incubation under continuous fluorescent light for 21 days at 20°C. If two lines of perithecia were produced (i.e. a line of perithecia borne by the test strain and another borne by the reference strain), the tested strain was considered female-fertile and of the opposite mating type to the reference strain. If a single line of perithecia was produced (sexual organs borne by the reference strain only), the tested strain was considered female-sterile and of the opposite mating type to the reference strain.

Marker name	Chromosome	Supercontig (Range on Supercontig pb)	Repeat motif	Primers sequences	T_m (°C)	N_t
pyrms63-64	1	9 (243439-243601)	CT_{15}	F: (NED)-TTGGGATCTTCGGTAAGACG R: GCCGACAAGACACTGAATGA	57	11
pyrms261-262	1	27 (1664544-1664755)	TA_{11}	F: (VIC)-ATTTCTTGGGTGCTTCGTTG R: CGAAGCGTGTTGAGAGTGAA	57	29
pyrms83-84b	2	18 (211631-211742)	TCA_{13}	F: (PET)-GTCTGCCTCGACTCCTTCAC R: GCAAAGTTGTTTGAGCAAGG	57	10
pyrms319-320	2	18 (52505-52795)	CAA_6	F: (NED)-TAAGACCACTGGCGGAATCT R: GGCTTTGTCTGGTTGTACGG	57	4
pyrms77b-78	3	24 (510594-510813)	CA_{24}	F: (PET)-AGGCTCTCTGCCTACGAAGT R: GCTTTCGGCAAGCCTAATC	57	14
pyrms685-686	3	28 (312164-312359)	TGC_{12}	F: (VIC)-AGAACGCCAATGGAGGAAG R: GCACACCGTCGTCTATACCC	57	9
pyrms607-608	3	28 (1095575-1095864)	GCA_{13}	F: (VIC)-CCCAAGCTCCATAATACGCTAC R: TCCGAGACTCTTTGGATAGCAC	57	9
pyrms37-38	4	15 (1042756-1042961)	CA_6+CT_{12}	F: (NED)-ACCCTACCCCCACTCATTTC R: AGGATCAGCCAATGCCAAGT	57	6
pyrms47-48	4	12 (707029-707203)	TA_{15}	F: (FAM)-TCACATTTGCTTGCTGGAGT R: AGACAGGGTTGACGGCTGAA	57	12
pyrms177b-178b	4	12 (1269705-1269803)	AC_{21}	F: (NED)-TCACAAGGATGGATTGCTTC R: CAGTTCGAGGTTAGCCGTTC	57	15
pyrms233-234	5	10 (38361-38617)	CAG_{10}	F: (FAM)-TGAGATGGACCGCATGATTA R: TTGATGGCAGAGACATGAGC	57	14
pyrms683b-684b	5	26 (781546-781761)	$CTAC_{18}$	F: (VIC)-TGGGTGCAGTGCAGTTTAAT R: TGCGGCTAACTGGCAACTAT	57	29
pyrms427-428	5	13 (331827-332045)	AT_{16}	F: (VIC)-CTGTCACCACAACCAAGACG R: TTGCCCTGATTTGTCAGTCA	57	17
pyrms221c-222c	6	11 (229752-229917)	$GCAG_7$	F: (NED)-CCGTTTTTCGCGTTATTGTT R: GTCGGTTGACGACTCATTCC	57	8
pyrms637b-638b	6	21 (1709549-1709688)	ATC_{10}	F: (NED)-CATCACTGTCCAACAACTCCA R: TCAGGAATTCCCTCATGACA	57	3
pyrms657-658	6	21 (3745780-3745947)	CA_{12}	F: (VIC)-ATCAGTCGAACCCACAAAGC R: ATGTGTGGACGAACCAGTCC	57	4
pyrms385-386	7	23 (2709066-2709228)	TAG_9	F: (VIC)-CCTTGTTTTCCCCCTGTGTA R: TGGGAAGAAGAGACCGAAGA	57	9

Supplementary Table S5.1. Microsatellite primers. Marker name, position, repeat motif, primer sequence, melting temperature (T_m) and total number of alleles (N_t) for 17 microsatellite markers tested on all strains genotyped.

DNA extraction and microsatellite amplification

DNA was extracted as previously described (Adreit *et al.* 2007) and stored at -20°C. We genotyped all strains with 17 microsatellites developed for routine population genetics studies (Adreit *et al.* 2007), selected from a set of about 300 microsatellite markers developed for genetic mapping (Kaye *et al.* 2003; Sreewongchai *et al.* 2009; Wang *et al.* 2005). The chosen markers were evenly distributed on the seven *M. oryzae* chromosomes (Supporting information Table S5.1). The microsatellites were amplified by PCR (QIAGEN multiplex PCR kit) and the products obtained were separated and analyzed on a 16-capillary ABI Prism 3130XL machine (Applied Biosystems). For this analysis, we mixed 1 µl of amplified products with 15 µl Formamide GeneScan-500LIZ size marker (Applied Biosystems, Foster City, CA). GENEMAPPER® (Applied Biosystems) was used for allele calling and assignment. The reproducibility of genotyping between runs was ensured by the inclusion of two control strains in each run. Individuals with more than three missing data were eliminated, leaving 406 strains for use in the population genetics analyses.

Genetic diversity and clonal structure

The mean number of alleles per locus, N_a, and gene diversity, H_e (Nei 1987), were calculated for each population with GENEPOP 4 (Raymond & Rousset 1995). H_e, which is frequently used to estimate expected heterozygosity, provides a measurement of unbiased gene diversity (the probability that two alleles chosen at random from a sample are identical, regardless of sample size). It is therefore suitable for use even with haploid organisms. The number of MLG (*G*) and the *G:N* ratio (*N* = number of genotyped strains) were calculated for each population with MULTILOCUS 1.3 (Agapow & Burt 2001). Differences between two MLGs concerning only one of the 17 loci are more likely to have arisen through mutation than through recombination. We accounted for this by considering two MLGs identical at all but one locus to belong to the same clonal group (*CG*). We used eBURST 3.8 (Feil *et al.* 2004) to detect MLGs differing at only one locus and to group these MLGs into the same clonal group. The *CG:N* ratio was also calculated, to determine the fraction of the sample consisting of different MLGs, corrected for mutation. As *CG:N* could be biased by differences in sample size between the nine populations (Grünwald *et al.* 2003), we also estimated Stoddart and Taylor's genotypic diversity index *STG* (Stoddart & Taylor 1988). This index ranges from 1 in clonal populations to *N* (sample size) in recombining populations. Grünwald *et al.* (2003) suggested that the best method of scaling was to divide *STG* by the expected maximum

number of MLGs (g_{max}). However, the large differences in allelic diversity between the populations studied here tended to bias $g_{max.}$ We therefore standardized *STG* by dividing by sample size.

Statistical tests were carried out with the R package version 2.12.0 (R Development Core Team, 2010).

Multilocus and pairwise linkage disequilibrium

Multilocus LD was estimated by calculating the association index $\overline{r}_D$ in MULTILOCUS 1.3 (Agapow & Burt 2001). The widely used association index I_A is based on the variance of the distances between all pairs of individuals (the number of loci by which each pair of individuals differs) and was developed to estimate the degree of deviation from random mating in a population (but see De Meeûs & Balloux 2004 for a discussion on the limitations of this index for detecting recombination). $\overline{r}_D$ is derived from I_A, a slight modification rendering it independent of the number of loci scored (Agapow & Burt 2001). It varies between 0 (random mating) and 1 (complete linkage disequilibrium). Multilocus and pairwise $\overline{r}_D$ were calculated from data corrected for clonal group (see above). Their significance was assessed on the basis of 1000 randomizations. The significance of allelic associations between pairs of loci was also assessed by carrying out Fisher's exact test with GENEPOP 4 software (Raymond & Rousset 1995). Finally, we used MULTILOCUS 1.3 to calculate the proportion of pairs of loci that were compatible (four-gamete test adapted for multiple alleles).

Computer simulations

It is not straightforward to determine whether recombination occurs in a population on the basis of summary statistics, partly because these statistics are affected by factors other than recombination. They also reflect historical population structure and current rates of mutation, drift and migration (De Meeûs and Balloux 2004). We assessed the likelihood of the values of $\overline{r}_D$ and *CG:N* obtained for the populations studied being generated in the absence of recombination, by simulating asexual populations evolving in conditions generating levels of genetic diversity similar to those observed. In particular, we investigated the effects of high effective population size and migration. Simulations were carried out with quantiNEMO 1.0.2 software (Neuenschwander *et al.* 2008), modified to account for asexuality. We simulated asexual individuals characterized by 17 microsatellite loci following a stepwise mutation

model (SMM), with a mutation rate $\mu = 10^{-5}$. The simulations were designed to be compatible with the high level of genetic diversity observed. Such high diversities could be accounted for by high effective population sizes. We tested two values for effective population size: N_e = 1000 and N_e = 10000 individuals. Migration would also be expected to increase within-population diversity. The spores of *M. oryzae* have a limited dispersal range (Nottéghem 1977), and natural populations from neighboring fields are generally highly structured in invaded areas (Tharreau *et al.* 2009). However, little is known about the scale and intensity of gene flow between populations in the putative centre of origin of this species. We ensured that our simulations were conservative, by simulating nine populations connected by migration. For each parameter set, we simulated nine populations of equal size, connected to each other by migration, in an island model with a migration rate $m = 0.5$. The stepping-stone model of migration and a higher mutation rate ($\mu = 10^{-4}$) were also tested and yielded similar results (not shown). Only simulations with $\mu = 10^{-5}$ and $N_a = 25$ are shown here.

For each simulation, we followed the establishment of an equilibrium between mutation and drift. We sampled 30 individuals, at random, from each population, every 1000 generations and used them to estimate H_e for each population. In this way, we were able to demonstrate the stabilization of H_e at the end of each simulation (i.e., 60 000 generations). At this time point, we sampled 30 individuals at random from each simulated population, for the estimation of *CG:N* and $\overline{r_D}$ (with clone correction). The mean and standard deviation of *CG:N* and $\overline{r_D}$ were then calculated over 18 simulated populations (two replicates of nine populations).

Simulations were also performed to follow changes in the number of MLGs and pairwise LD between two consecutive clonal generations. We generated datasets for the 17 microsatellite markers considered for nine simulated clonal populations of 1000 individuals each. We used a high mutation rate ($\mu = 10^{-4}$) and a migration rate of $m = 0.5$ (island model). Once an equilibrium between mutation, migration and drift was reached, we sampled 24 individuals from two consecutive generations within each of the simulated populations. The results for these nine simulations were compared with real data for the CH1 population. We accounted for differences in sample size between the CH1-2008 and CH1-2009 populations by performing 30 random resamplings of 24 individuals in the 2009 population. We determined the number of multilocus genotypes and the number of clonal groups identical in the two consecutive generations (for simulated datasets) or years (for real resampled datasets).

We also assessed the significance of the linkage disequilibrium between pairs of loci, in each sample, for each generation (for simulated datasets) or year (real resampled datasets), by carrying out Fisher's exact test in GENEPOP 4 (Raymond & Rousset 1995). Bonferroni correction was applied to adjust the significance threshold for multiple testing (136 possible pairs). We then calculated the percentage of pairs in linkage disequilibrium maintained between two consecutive generations or years.

Results

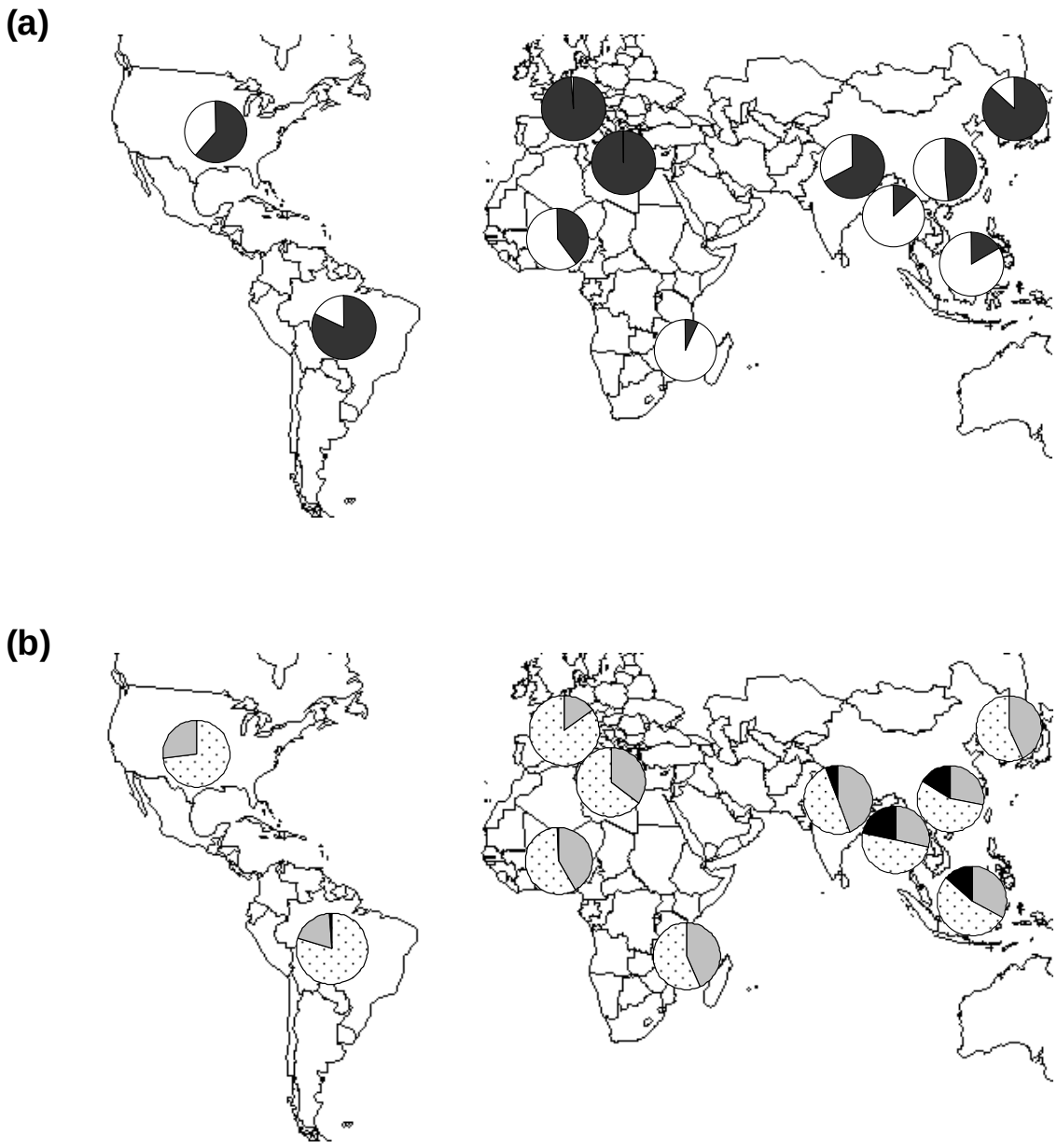
Geographic distribution of mating type and female fertility

We analyzed the worldwide geographic distribution of mating types of 3800 *M. oryzae* strains (collected as single isolates from different locations) and that of female-fertile strains for a subset of 2805 strains. Both mating types were found in most regions of the World, with the exception of the Mediterranean region (MAT1 strains only; Supporting information Fig. S5.1a). We identified a total of 227 female-fertile strains, 225 of which were sampled in Asia. Only two female-fertile strains were identified outside South Asia, both in South America (French Guiana). Thus, female-fertile strains were present almost exclusively in Asia, and their frequencies were highest close to the Himalayas (204/225 female-fertile strains; Supporting information Fig. S5.1b). Moreover, the two mating types were equally frequent in this region, identifying the Himalayan foothills as the best place to search for evidence of sexual reproduction in *M. oryzae*.

Biological evidence of sexual reproduction

Biological and population genetics investigations were conducted on population data (data for at least 20 strains isolated from the same field, on the same date). The relevant geographic scale for populations of *M. oryzae* is the rice field, because this species mostly disperses over short distances (a few meters; Nottéghem 1977). We characterized nine populations collected around the world (Fig. 5.1, Table 5.1): five from the Himalayan foothills and four from other continents (Europe, America and Africa).

Strains of the two mating types were found in all the Asian populations tested, and in the US population, whereas a single mating type was detected in the other populations (Fig. 5.1). Female-fertile strains were found only in Asian populations, but at different frequencies: <20% in the Chinese populations CH2, CH3 and CH4, and >50% in CH1 (2008 and 2009)



Supplementary Figure S5.1. Worldwide distribution of mating types and female fertility in a non-populational collection of *M. oryzae* strains from rice (samples collected at the continental scale). (a) Proportions of MAT1 (black) and MAT2 (white) in a collection of 3800 strains. (b) Proportions of female-fertile (black), female-sterile (grey) and completely sterile (dotted) strains in a subset of 2805 strains.

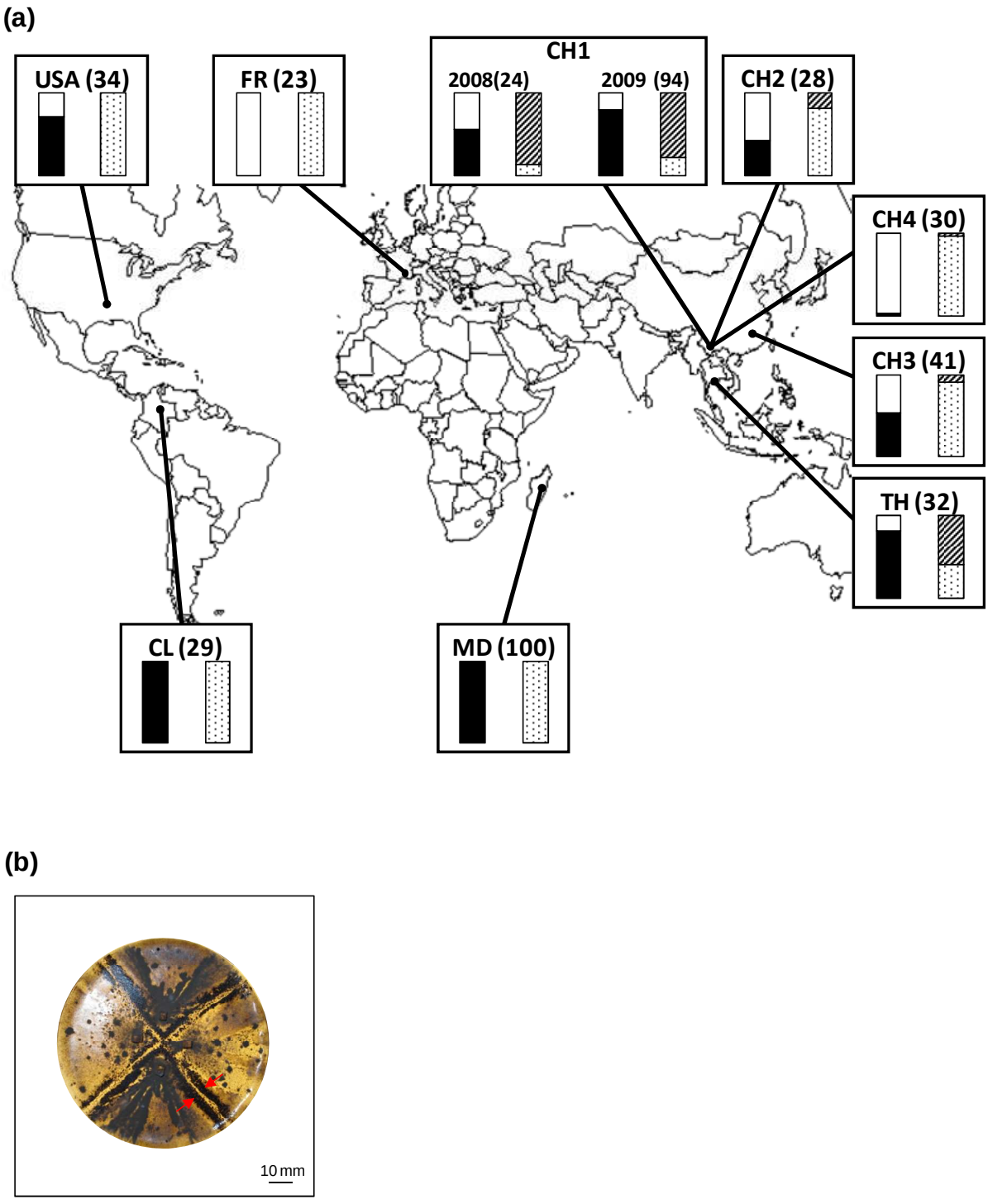


Fig. 5.1. Mating types and female fertility in nine *M. oryzae* populations. (a) Percentage of MAT1 (white) and MAT2 (black) strains and of female-fertile (hatched) and female-sterile (dotted) strains in each population, determined by crossing *in vitro*. The sample size is indicated in brackets. (b) Coculture of female-fertile strains of opposite mating types (here from the CH1 population) on a Petri dish. After 20 days of incubation at 20°C, two lines of perithecia (indicated by red arrows) are formed between the two strains.

and in the Thai population TH (Fig. 5.1). Thus, only the Asian populations had the biological characteristics required for sexual reproduction. Furthermore, for these populations, *in vitro* crosses between strains of opposite mating types from the same population (CH1-2008 or TH) led to the completion of the entire sexual cycle, including the production of viable progenies. In one of the crosses between two CH1 strains, we checked that the mating type locus and nine unlinked microsatellite markers followed the expected 1:1 segregation in the progeny (data not shown). All these offspring had different MLGs (data not shown).

Genetic signatures of recombination

We characterized the genetic diversity of all populations (Table 5.1), using 17 microsatellite markers. Considering all the individuals in the nine populations studied, the number of alleles at each locus varied from 3 (Pyrms637b-638b) to 29 (Pyrms261-262 and Pyrms 683B-684B; Supporting information Table S5.1). The genetic diversity of populations was assessed by calculating unbiased gene diversity (H_e) and the mean number of alleles per locus (N_a). Neither of these variables is affected by the mode of reproduction in haploid organisms (Balloux *et al.* 2003; Halkett *et al.* 2005). The populations studied clearly fell into two groups defined on the basis of these diversity indices (Table 5.1, Fig. 5.2a): CH1 (2008 and 2009), CH2, CH3, TH and USA clearly had a significantly higher gene diversity (Student's *t* test for H_e: $P = 0.01$) and a significantly larger number of alleles (Student's *t* test for N_a: $P = 6 \times 10^{-3}$) than the other four populations.

The degree of clonality, assessed by calculating *CG:N* and *STG:N*, differed significantly between populations (Table 5.1, Fig. 5.2b). With the exception of CH4, all populations from the putative centre of origin had a significantly higher *CG:N* than the other populations (mean = 58% vs. 13% in the other populations; Student's *t* test $P = 2 \times 10^{-5}$). Estimates of genotypic diversity based on the standardized Stoddart and Taylor's index *STG:N* were consistent with the results obtained with *CG:N*. Genotypic diversity exceeded 50% in the Asian populations CH1, CH2 and TH, whereas it was below 32% in CH4 and non-Asian populations. The CH3 population was intermediate, with a *STG:N* of 43%.

Consistent with these findings, in the populations from the putative centre of origin (other than CH4), multilocus linkage disequilibrium (LD) was weak, with an $\overline{r_D}$ index of 0.07 to 0.25 (vs. 0.30-0.62 in the other populations; Student's *t* test $P = 0.04$). Despite these significant differences between Asia and the other continents, $\overline{r_D}$ was significantly different

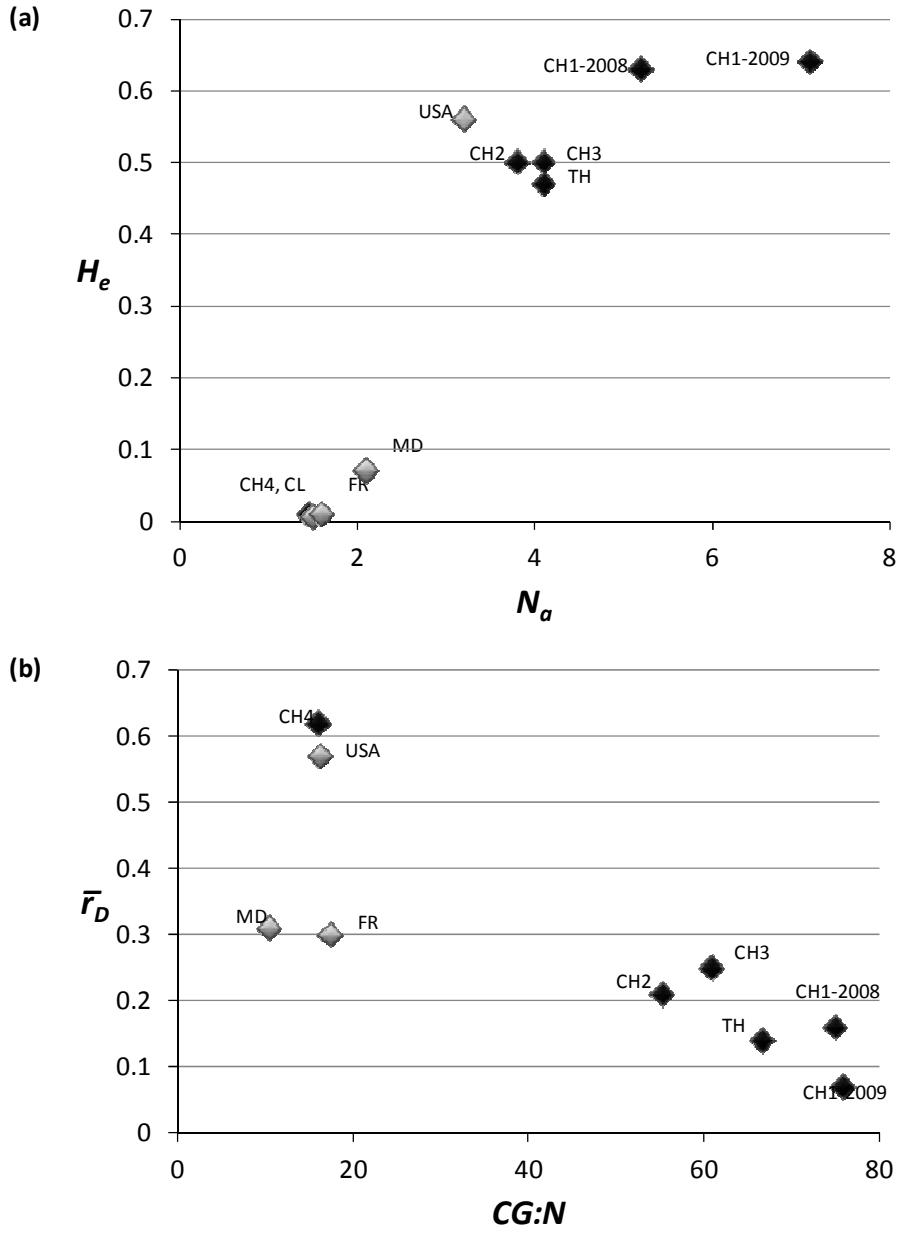
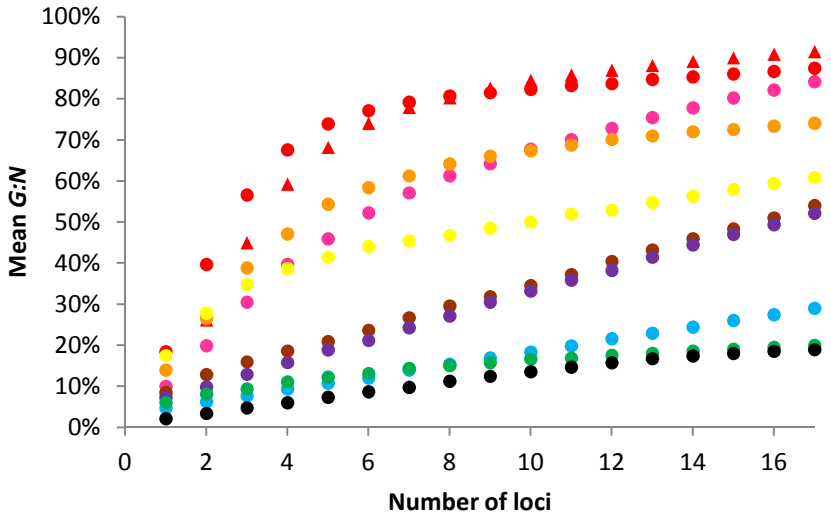


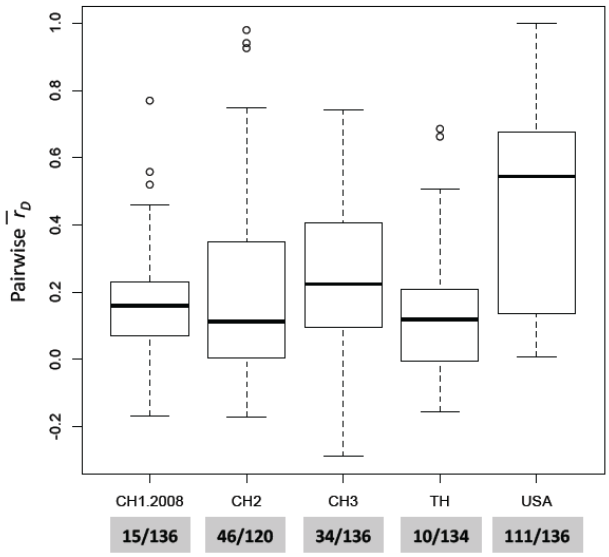
Fig. 5.2. Diversity and recombination in the 10 populations studied (Asian populations in black, populations from other parts of the world in grey). (a) Gene diversity, H_e as a function of mean allelic diversity, N_a. (b) Multilocus association index $\bar{r}_D$ (calculated from data corrected for mutation, see Methods), as a function of *CG:N* (expressed in %). The CL population is not represented on panel b because the multilocus $\bar{r}_D$ could not be estimated for this population.

from 0 in all populations ($P < 0.008$ after 1000 randomizations), making it possible to reject the hypothesis of panmixia. Differences in $\overline{r_D}$ between populations seemed to follow a more continuous distribution than the clearly bimodal distribution of *CG:N* values. This might reflect the sensitivity of $\overline{r_D}$ to factors other than recombination, such as migration and population size (De Meeûs & Balloux 2004). We also determined the number of loci required to discriminate between the various MLGs in the different populations, because sexual reproduction would be expected to generate sufficient allelic combinations for the detection of many MLGs with small numbers of loci (Delmotte *et al*. 2002). In populations from the putative centre of origin (other than CH4), the identification of 62 to 88% of the observed MLGs was possible with six of the 17 loci, whereas only 41 to 46% of MLGs could be distinguished with the same number of loci on the other populations (Supporting information Fig. S5.2). These results strongly suggest that recombination occurred in the CH1 (2008 and 2009), CH2, CH3 and TH populations.

Investigations of pairwise LD provide information about the intensity and extent of recombination in genomes. Pairwise $\overline{r_D}$ values were calculated for data corrected for mutation (see methods), for populations in which a sufficiently large number of pairs (i.e. non-monomorphic pairs) could be tested: the CH1, CH2, CH3, TH and USA populations. We also tried to investigate the pairs displaying significant association in these populations. The proportion of the 136 possible pairs displaying significant pairwise LD was low (7-38%) in the CH1-2008, CH2, CH3 and TH populations, but reached 82% in the USA population (Supporting information Fig. S5.3). Furthermore, pairwise $\overline{r_D}$ values were centred on 0.2 in the Asian populations tested, versus 0.6 in the USA population. By observing pairs of loci, it is also possible to infer whether the different genotypes observed result from mutation only (compatible pairs; see methods). All pairs of loci would be expected to be compatible in clonal populations, whereas very few pairs of loci would be expected to be compatible in recombining populations. We found that 97 to 100% of the pairs of loci were compatible in the CH4, FR, MD and USA populations. The proportion was lowest in the CH1 population (27% in 2008 and 1% in 2009) and intermediate in the TH, CH2 and CH3 populations (47 to 83%). The USA population is a good asexual “control” population for comparison with the Asian populations, avoiding the confounding effect of genetic diversity. Indeed, none of the strains sampled from this population was female-fertile, but the genetic diversity of this population (as assessed by determining H_e and N_a) was similar to that of the Asian



Supplementary Figure S5.2. Relationship between *G:N* and the number of scored loci in the different populations. For each population, the mean values of *G:N* (number of unique multilocus genotypes divided by sample size) is plotted against the number of scored loci, *x*, chosen randomly from the 17 studied. For each value of *x*, the mean *G* is calculated, for each population, after 1000 randomizations. Red: CH1 (2008: circles, 2009: triangles). Pink: CH2. Yellow: CH3. Green: CH4. Orange: TH. Blue: CL. Purple: FR. Brown: USA. Black: MD.



Supplementary Figure S5.3. Pairwise linkage disequilibrium in five populations. Boxplots of values of pairwise $\bar{r}_D$ (calculated with MULTILOCUS 1.3 from data corrected for mutation) were drawn for the five populations in which a sufficient number of locus pairs could be tested (maximum 136 pairs). For each population, the grey box indicates the number of locus pairs that were significantly linked (determined with Fisher's exact test procedure implemented in GENEPOP 4) over the total number of pairs tested. The other populations were not considered, because only 10 to 45 pairs could be tested due to the large number of monomorphic loci.

populations. This diversity resulted from the coexistence of genetically unrelated clones within the USA population, probably due to multiple introductions of strains of different origins. The indices used to detect recombination (*CG:N*, $\overline{r}_{D,}$ pairwise $\overline{r}_{D}$, and proportion of compatible pairs) clearly distinguished the USA population from the Asian populations (with the exception of CH4; Table 5.1). These results confirm that the non-Asian and CH4 populations have the genetic features expected of populations displaying clonal reproduction, whereas most Asian populations, including CH1 in particular, have genetic characteristics compatible with sexual reproduction.

Evidence of sexual reproduction provided by computer simulation

We investigated whether the values of *CG:N* and $\overline{r}_{D}$ observed in most Asian populations could be reached without recombination, by simulating clonal populations connected to each other by migration, with a migration rate m = 0.5, for 17 microsatellite loci with a mutation rate $\mu = 10^{-5}$ (Supporting information table S5.2). The genetic diversity of these simulated clonal populations (N_a and H_e) was similar to that observed for the Asian populations. Even with a high effective population size (N_e = 10 000), which would probably decrease the effects of genetic drift, the *CG:N* index reached at equilibrium was always significantly lower (14-43%, Kruskal-Wallis test $P = 6 \times 10^{-3}$) than that for the Asian populations. Consistently, $\overline{r}_{D}$ was always significantly higher (0.33-0.40, Kruskal-Wallis test $P = 3 \times 10^{-3}$) in the simulated populations than in the Asian populations. In the TH and CH1 populations, the observed values of *CG:N* (67 % and 75%, respectively) and $\overline{r}_{D}$ (0.14 and 0.16, respectively), were very different from the values obtained in simulations (*CG:N* = 14% ± 4% and 50% ± 8% for N_e = 1000 and 10 000, respectively; $\overline{r}_{D}$ = 0.33 ± 0.12 and 0.40 ± 0.18 for N_e = 1000 and 10 000, respectively; Fig. 5.3, Tables 5.1 and S5.2). The observed values for the CH2 and CH3 populations were less different from the values obtained in the simulations: *CG:N* = 55 % and 61%, respectively and $\overline{r}_{D}$ = 0.21 and 0.25, respectively. The simulations showed that the high *CG:N* and low $\overline{r}_{D}$ values obtained for most of the populations from the putative centre of origin of the species were unlikely to have been achieved by strict asexuality, supporting the notion that sexual reproduction occurs in Asia.

Simulation	Replicate	*G*	*CG*	*CG:N*	N_a	H_e	$\bar{r}_D$	*Comp*
	1	9	7	23.3%	1.7	0.17	0.16	95.6%
	2	8	4	13.3%	1.6	0.14	0.25	100%
	3	10	6	20.0%	1.8	0.17	0.14	97.8%
	4	7	5	16.7%	1.6	0.17	0.15	100%
	5	8	6	20.0%	1.6	0.14	0.24	97.1%
	6	5	4	13.3%	1.5	0.14	0.27	100%
	7	7	5	16.7%	1.6	0.18	0.32	100%
	8	6	4	13.3%	1.5	0.14	0.36	100%
	9	7	5	16.7%	1.6	0.18	0.20	100%
N_e = 1,000	10	6	3	10.0%	1.5	0.16	0.37	97.8%
	11	7	3	10.0%	1.5	0.14	0.43	98.5%
	12	4	3	10.0%	1.4	0.14	0.41	100%
	13	5	3	10.0%	1.4	0.14	0.51	100%
	14	5	3	10.0%	1.5	0.13	0.40	100%
	15	5	3	10.0%	1.5	0.16	0.51	100%
	16	6	3	10.0%	1.5	0.15	0.44	100%
	17	5	3	10.0%	1.5	0.13	0.40	100%
	18	7	3	10.0%	1.5	0.14	0.43	100%
	mean±sd	6.5±1.6	4.1±1.3	13.5±4.3%	1.5±0.1	0.15±0.02	0.33±0.12	99.3±1.3%
	1	22	16	53.3%	4.2	0.50	0.24	39.0%
	2	22	15	50.0%	3.4	0.47	0.14	43.4%
	3	24	15	50.0%	4.4	0.46	0.24	43.4%
	4	19	12	40.0%	3.2	0.48	0.16	50.0%
	5	20	13	43.3%	4.3	0.55	0.28	64.7%
	6	18	13	43.3%	4.3	0.53	0.21	53.7%
	7	19	14	46.7%	4.2	0.58	0.38	52.2%
	8	19	13	43.3%	3.4	0.48	0.15	48.5%
	9	18	12	40.0%	4.2	0.46	0.27	51.5%
N_e = 10,000	10	23	16	53.3%	5.1	0.68	0.55	66.2%
	11	23	16	53.3%	4.9	0.68	0.61	72.1%
	12	26	16	53.3%	4.8	0.66	0.53	72.1%
	13	25	18	60.0%	5.1	0.69	0.59	69.9%
	14	24	19	63.3%	5.3	0.70	0.53	61.0%
	15	23	18	60.0%	5.0	0.72	0.55	79.4%
	16	20	13	43.3%	4.9	0.66	0.61	87.5%
	17	21	14	46.7%	5.0	0.70	0.56	79.4%
	18	26	19	63.3%	5.4	0.68	0.51	64.7%
	mean±sd	21.8±2.6	15.1±2.3	50.4±7.7%	4.5±0.7	0.59±0.10	0.40±0.18	61.0±14.1%

Supplementary Table S5.2. Genetic diversity and multilocus linkage disequilibrium in the simulated populations. For the two conditions (*Ne* = 1000 and *Ne* = 10 000), 18 replicates were performed. Mutation rate and migration rate were set at $\mu = 10^{-5}$ and $m = 0.5$, respectively. Indices were calculated after 60 000 generations, for a sample of 30 individuals for each replicate. Mean values and standard deviation over the 18 replicates are given for each set of conditions. *G*: number of multilocus genotypes. *CG*: number of clonal groups (calculated with e-Burst, see methods). *CG:N*: proportion of clonal groups. N_a: mean number of alleles per locus. H_e: gene diversity. $\bar{r}_D$: multilocus association index, calculated from data corrected for mutation (see methods). *Comp*: Proportion of compatible pairs of loci. $\bar{r}_D$ was significantly different from 0 in all populations (as assessed with MULTILOCUS 1.3 after 1000 randomizations).

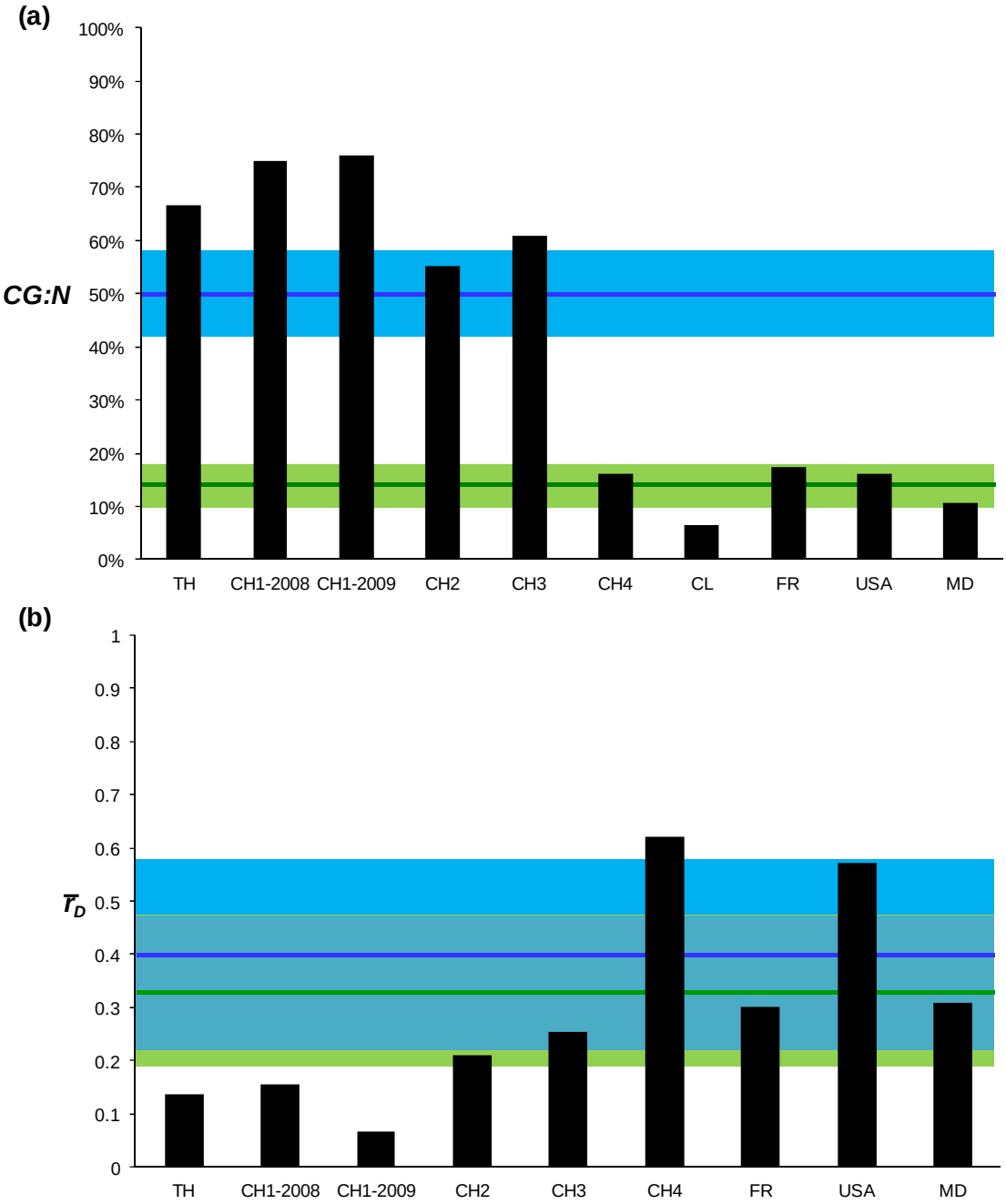


Fig. 5.3. Genotypic diversity and linkage disequilibrium in simulated clonal populations; comparison with observed data. (a) *CG:N* in each studied population (bars). (b) Multilocus $\bar{r}_D$ (calculated from data corrected for mutation, see methods) for each of the populations studied except for CL (bars). For simulated populations, two replicates of nine simulated clonal populations with a mutation rate $\mu = 10^{-5}$, an initial $N_a = 25$, a migration rate $m = 0.5$ (island model of nine connected populations) and a population size $N_S = 1000$ (green) or $N_S = 10\ 000$ (blue), were studied. *CG:N* and $\bar{r}_D$ were calculated on random samples of 30 individuals taken from each of the 18 simulated populations after 60 000 generations (lines and filled areas representing the mean and standard deviation, respectively).

Resampling a sexually reproducing population

The CH1 population collected in 2008 provided the best evidence for contemporary sexual reproduction, with the two mating types present at similar frequencies, a high frequency of female-fertile strains (79.2%; Fig. 5.1), the highest value of *CG:N* and the lowest level of LD (*CG:N* = 75%, $\overline{r_D}$ = 0.16; Table 5.1, Fig. 5.2b). The sample collected from the same field in 2009 presented similar characteristics, indicating the occurrence of sexual reproduction (Fig. 5.1, Table 5.1, Fig. 5.2b). The 2008 and 2009 samples of the CH1 population were not differentiated (F_{ST}=0.006), confirming that they belonged to the same population. Sexual reproduction, by reshuffling allelic combinations at different loci, would be expected (i) to generate new MLGs across generations, and (ii) to disrupt significant associations between pairs of loci. Conversely, in strictly clonal populations, we would expect (i) MLGs to persist across generations, and (ii) the persistence of significantly linked pairs of loci across generations. A larger number of MLGs persisted across two simulated consecutive generations in the clonal simulations (7.2 ± 1.9 standard deviation) than between the two samples of the CH1 population (in which one MLG persisted). These results are consistent with significant allele shuffling between the two years. We then used the same simulated data to focus on changes in statistical associations between pairs of loci between the two sampled years. The percentage of locus pairs disrupted between two consecutive generations (NS/S) and the percentage of pairs linked only in the second generation (NS/S) were not significantly different in the simulated populations and the CH1 population (Fig. 5.4). However, the percentage of pairs remaining significantly linked in both generations (S/S) was significantly lower in the CH1 population (0.4% ± 0.5% standard deviation) than would be expected under strict asexual reproduction (25% ± 8% standard deviation; Student's *t* test P = 3.10^{-5}). Conversely, pairs of loci remaining significantly unlinked (NS/NS) were the most frequent in the CH1 population, accounting for a mean of 89% (± 2% standard deviation) of the pairs. This proportion was significantly lower in simulated clonal populations (mean of 46% ± 9% standard deviation; Student's *t* test P = 6.10^{-8}). Thus, the CH1 population deviates significantly from the null hypothesis of an absence of recombination, and this deviation is unlikely to be due to sampling bias or to other factors, such as high mutation rates or migration.

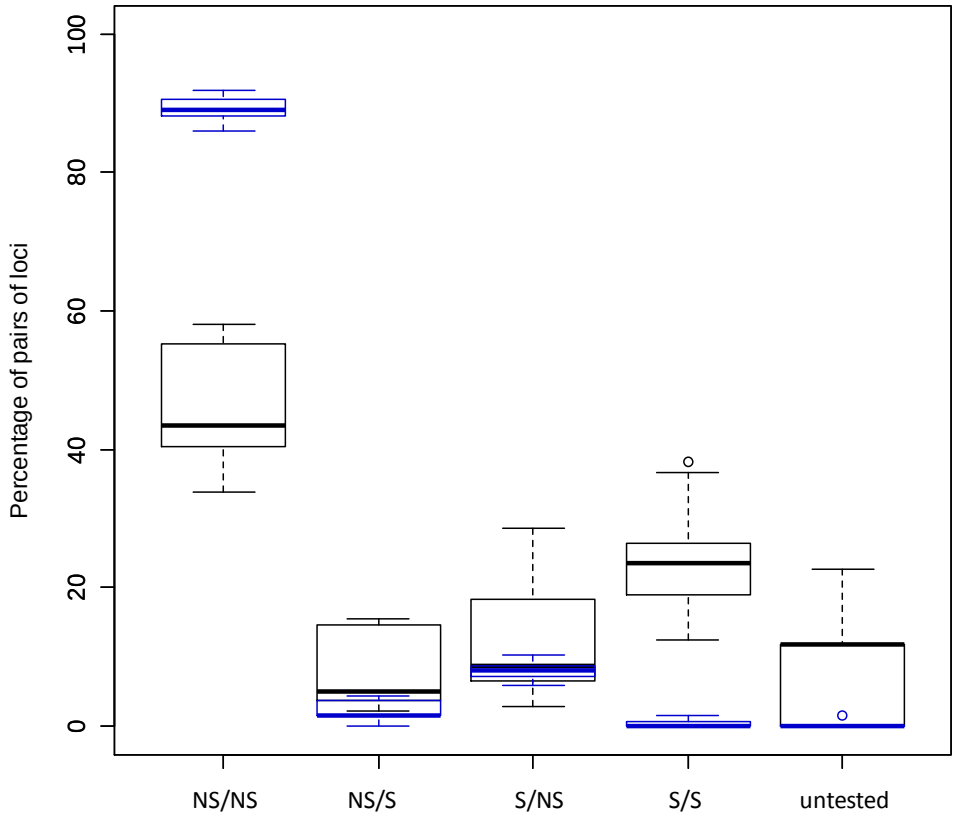
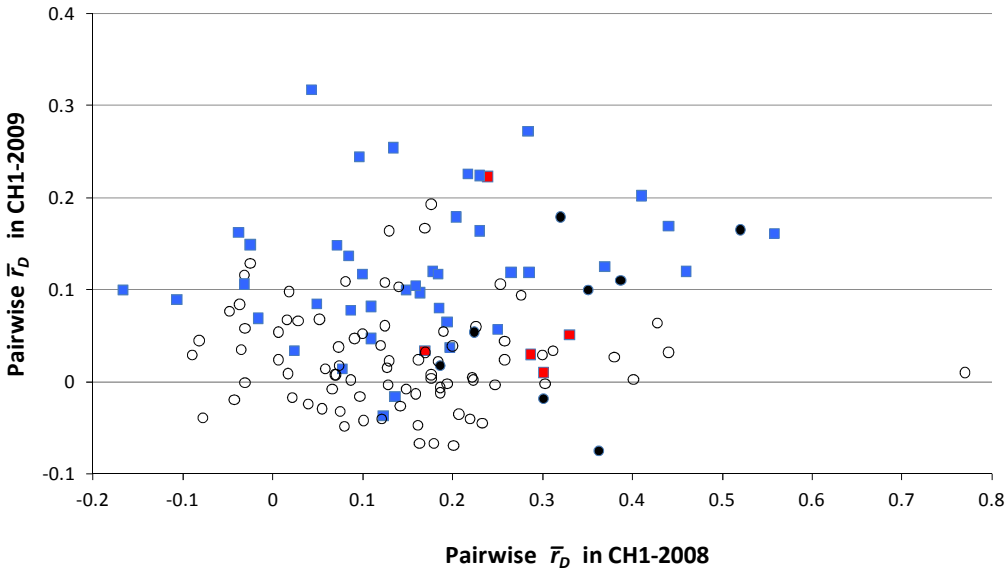


Fig. 5.4. Comparisons of pairwise LD between two consecutive generations in simulated clonal populations and in the CH1 population. The different categories on the x-axis represent pairs of loci not significantly linked in the two consecutive years (NS/NS), not significantly linked in the first year but significantly linked the following year (NS/S), significantly linked in the first year but not in the following year (S/NS), significantly linked in the two consecutive years (S/S), and untested. White boxplots represent the percentage of locus pairs in each category calculated from random samples of 24 individuals from two consecutive generations in nine simulated clonal populations of 1000 individuals each, at mutation-migration-drift equilibrium. Blue boxplots represent the percentage of locus pairs in each category calculated from the CH1-2008 sample and 30 random resamplings of 24 individuals from the CH1-2009 sample.



Supplementary Figure S5.4. Pairwise linkage disequilibrium between two consecutive years in the CH1 population. Values of pairwise $\bar{r}_D$ (calculated with MULTILOCUS 1.3 from data corrected for mutation) in 2009 are plotted against 2008 values. Black circles represent the pairs significantly associated in both 2008 and 2009; red squares represent the pairs significantly associated in 2008 but not in 2009; blue squares represent the pairs significantly associated in 2009 but not in 2008; white circles represent the pairs not significantly associated in either year.

Moreover, no significant correlation was observed between the 2008 and 2009 values for pairwise LD, as estimated by calculating the pairwise $\overline{r_D}$ (Spearman's rho non-parametric test: $P = 0.39$; Supporting information Fig. S5.4), confirming a lack of maintenance of pairwise associations over time in this population. These results provide strong evidence for genome-wide recombination in the CH1 population between the two sampling years.

Discussion

This study is the first to combine several different, complementary approaches (reproductive biology, population genetics and computer simulations) to explore possible differences in the mode of reproduction of *M. oryzae* between populations at its centre of origin and in invaded areas. Contemporary sexual reproduction has never previously been unequivocally demonstrated for this species. The most successful previous attempt at demonstrating contemporary sexual reproduction in *M. oryzae* was the study by Kumar *et al.* (1999). Using the multilocus association index I_A, these authors identified two populations from North India with no significant LD. However, this result was not confirmed with samples collected the following year. The absence of LD observed in the first year of sampling may reflect recent or ancient recombination. Alternatively it may simply result from sampling bias, as recent studies have shown that I_A is sensitive to evolutionary forces other than recombination (such as genetic drift and migration; De Meeûs & Balloux 2004) and that potential bias in sampling is likely to mimic the recombination signature detected by measuring LD (Prugnolle & De Meeûs 2009). Such biased sampling is likely to have occurred in Kumar's study, because samples of different rice varieties (and sometimes from different years) were pooled for analysis, and *M. oryzae* population structure depends strongly on rice variety (Chen *et al.* 1995).

Our analysis of a worldwide strain collection confirmed previous observations (Zeigler 1998; Tharreau *et al.* 2009) that strains of both mating types are found in Asia, and that female-fertile strains are almost entirely restricted to this area. Only two female-fertile strains were found outside this region, both in French Guiana. However, these strains are unlikely to reflect the presence of contemporary sexual reproduction, as historical and genotypic data indicate that these strains were probably imported from South Asia by Hmong migrants in the 1970s (J. L. Nottéghem pers. com.). This study represents a step forward by

confirming the co-occurrence of both mating types and female-fertile strains in Asia at the population scale. The presence of these features in populations from the Himalayan foothills is indicative of the occurrence of sexual reproduction in this region (Taylor *et al.* 1999; Zeyl 2009).

In addition to biological data, we used several population genetics tools to test for the occurrence of recombination, for further characterization of the mode of reproduction of the populations studied. All our analyses were consistent with recombination in the CH1, CH2, CH3 and TH populations, with the strongest evidence obtained for the CH1 population. Simulations of clonal populations with parameter ranges likely to maximize genetic diversity (i.e. high allelic diversity, large population size and high migration rate), showed that the low values of $\overline{r_D}$ and the high values of *CG:N* observed in the Asian populations could not be attained with a strictly clonal mode of reproduction. The CH1 population, in particular, had the highest *CG:N*, the highest genotypic richness and the lowest $\overline{r_D}$ with respect to simulated clonal populations and the most diverse clonal population sampled (USA). For the CH1 population, which was collected in the Yunnan province of China, we also investigated whether recombination was still occurring, by comparing samples collected in two consecutive years. We used the simulated clonal datasets to test the null hypothesis of the persistence of MLGs and significant pairwise allelic associations between two consecutive generations in the absence of recombination, even in the presence of high levels of genetic diversity. A single MLG was sampled in both years, and less than 1% of pairs of loci remained linked in both years, strongly suggesting that sexual reproduction is ongoing in the CH1 population. Signatures of recombination in Asian populations might also have resulted from parasexuality. This process is thought to occur in *M. oryzae* (Zeigler *et al.* 1997), but should generate only rare recombination events. It is therefore unlikely to have generated the reshuffling of alleles observed between 2008 and 2009 in the CH1 population. Sexual reproduction is thus the hypothesis most likely to account for all of the biological and genetic characteristics of the CH1 population. This work provides strong, albeit indirect evidence that sexual reproduction occurs in this species in Asia. The discovery of perithecia in the field would provide definitive evidence for contemporary sexual reproduction in the population concerned. However, the discovery of sexual structures would provide no information about the relative importance of the sexual and asexual modes of reproduction. For the management of control strategies, it is actually more important to determine the extent to which recombination occurs in the field than to find sexual stages of the fungus. It remains possible

that *M. oryzae* reproduces sexually outside of the growing season, on rice straw or a secondary host plant.

The detection of sexual reproduction, and hence recombination, in field populations of plant pathogens is important, because the choice of control strategy, particularly as concerns the use of resistant varieties and pesticides, depends largely on the reproduction system of the pathogen (McDonald & Linde 2002). Recombination increases the probability of favorable allelic combinations being generated, selected, and rapidly spread, thereby compromizing classical control strategies (McDonald & Linde 2002). Most of the strategies based on varietal resistance commonly proposed for the control of rice blast fungus (e.g. lineage exclusion and pyramiding; Bonman *et al.* 1992; Zeigler *et al.* 1994) are likely to fail if the pathogen displays sexual reproduction (McDonald & Linde 2002). For example, the lineage exclusion strategy is based on the observation that isolates from the same clonal lineage have genes controlling cultivar specificity (avirulence genes) in common. It has been suggested that the use of combinations of different resistance genes effective against different lineages might make it possible to exclude all lineages (Zeigler *et al.* 1994). This strategy implicitly assumes that there is no genetic exchange between lineages, because such exchanges would generate new combinations of avirulence genes, resulting in multivirulent strains, which would then be selected. Our results suggest that there is a need to reconsider blast management strategies, at least in some areas of South Asia. Variety management based on the use of different specific resistance genes at different times and in different areas may be preferable to accumulating multiple resistance genes in a single variety (pyramiding). Varieties with non-specific resistance could also be used, either as an alternative strategy or in combination with pyramiding, although the gradual breakdown of this type of resistance is also possible (McDonald & Linde 2002). Integrated management, based on various control methods (agronomic, genetic, chemical), could also be useful, to decrease selection pressure and slow adaptation.

This study provides new information about the centre of origin of *M. oryzae*. Asian cultivated rice, *Oryza sativa* (Londo *et al.* 2006), diversified and was domesticated in two centers, in southern China and northern India. Previous studies have identified the Himalayan foothills as a centre of genetic diversity for the *M. oryzae* strains pathogenic on rice (Zeigler 1998, Kumar *et al.* 1999, Tharreau *et al.* 2009). Populations from the centre of origin of a species are generally expected to be more diverse than populations from invaded areas, because migration from the origin is often accompanied by bottlenecks or founder effects,

leading to a loss of diversity (Sakai *et al*. 2001; Dlugosch & Parker 2008). Our findings confirm that gene diversity and allelic richness are greater in populations from Asia than in populations from elsewhere. The occurrence of sexual reproduction exclusively in this region also provides additional evidence that Asia is the centre of origin for this species. The USA and CH4 populations seem to constitute exceptions with particular demographic histories. Gene diversity was high in the USA population, whereas genotypic diversity was low, consistent with the presence of a mixture of clones, probably due to multiple introductions (Tharreau *et al.* 2009). By contrast, the gene diversity of the CH4 population was lower than that of other Asian populations, probably due to a bottleneck resulting from selection by the host. However, further population genetics studies on worldwide collections are required to confirm that Asia is the centre of origin of rice blast disease.

Our results raise questions about the evolution of sexual reproduction during the spread of pathogens outside their centre of origin. According to the general theory of sex evolution, sexual reproduction is likely to be selected in heterogeneous environments (Lenormand & Otto 2000; Otto & Lenormand, 2002). In the context of host-pathogen coevolution, sexual reproduction of the host is thought to be favored by strong parasitic pressure exerted by a diverse parasite population, as this reproductive system allows the recombination of defense systems to combat parasites (Hamilton *et al*. 1990; Otto & Michalakis 1998; Lively 2010). Conversely, pathogens are also likely to maintain sexual reproduction in areas of high host genetic diversity (Stukenbrock & McDonald 2008). In particular, for fungi making extensive use of sexual reproduction, theoretical models predict that, regardless of mating types ratios, female fertility may be more frequent in native (diverse) than in agricultural (more uniform) environments (Leslie & Klein 1996). Our study provides experimental support for these predictions, because female-fertile strains and sexual reproduction were maintained in areas in which diverse traditional varieties are grown. By contrast, asexual reproduction was observed in fields of highly uniform rice cultivars. These findings are consistent with the hypothesis that sexual reproduction is maintained only when the pathogen is confronted with diverse host populations. A loss of sexual reproduction in homogeneous environments has been documented in various other species, including aphids (Gilabert *et al.* 2009, Simon *et al.* 2010) and rotifers (Becks & Agrawal 2010). *Phytophthora infestans* (Gomez-Alpizar *et al.* 2007) and *Puccinia striiformis f.sp. tritici* (Bahri *et al.* 2009, Mboup *et al.* 2009, Ali *et al.* 2010) provide additional examples of Oomycete or fungal pathogens that have lost the ability to reproduce sexually in invaded agrosystems. By contrast,

other well studied fungal pathogens, such as *Mycosphaerella graminicola* (Linde *et al.* 2002), *Mycosphaerella fijiensis* (Rivas *et al.* 2004), *Phaeosphaeria nodorum* (Stukenbrock *et al.* 2006), *Ustilago scitaminea* (Raboin *et al.* 2007) and *Venturia inaequalis* (Gladieux *et al.* 2008), have retained both sexual and asexual reproduction systems, even in uniform agrosystems. In these species, the sexual cycle is essential for overwintering (*M. graminicola*, *P. nodorum*, and *V. inaequalis*), dissemination (*M. fijiensis*), or infection (*U. scitaminea*). The requirement of sexual reproduction for these processes may account for the conservation of sexual reproduction by these species outside of their native area, despite the relative uniformity of the invaded environments. These fungal pathogens demonstrate that sexual reproduction may be retained in individual species due to various short-term constraints (Gouyon 1999).

The evidence of sexual reproduction in Asia also raises questions about the maintenance of sexual reproduction in centres of origin. It remains unclear whether sexual reproduction confers a short-term ecological advantage on *M. oryzae* in native areas. It may provide the pathogen with a means of overwintering. The asexual spores of *M. oryzae* are not very resistant and perithecia are probably more resistant to adverse conditions. Fruiting bodies are known to serve as overwintering structures in some fungi (Agrios 1997). In *M. oryzae*, sexual reproduction would be maintained in traditional agro-ecosystems, to enable the pathogen to survive the winter, when there is no rice in the field, and to produce a source of primary inoculum for the following season. In more intensive agro-ecosystems, infected seeds harvested and stored by humans may allow the pathogen to overwinter and serve as a primary source of inoculum. Whatever the selection pressure, our results provide experimental evidence that changes in agro-ecosystems can have a major impact on the evolution of the reproduction system of associated plant pathogens.

Fungal pathogens of cultivated plants provide us with a unique opportunity to study the evolution of sex because (i) a single species may use several reproductive strategies, and (ii) evolutionary changes may occur faster in agro-ecosystems than in natural ecosystems, due to high reciprocal selection pressures (McDonald & Linde 2002; Stukenbrock & McDonald 2008). *M. oryzae*, with its coexistence of sexual and asexual populations, thus joins other well studied species, including aphids (Simon *et al.* 2010) and freshwater snails (Lively & Jokela 2002; Lively 2010), as an excellent model for this field of research.

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2. Analyses supplémentaires

En plus des travaux présentés dans l'article ci-dessus, nous avons réalisé des analyses supplémentaires pour appuyer l'existence de reproduction sexuée dans la population CH1.

Nous avons cherché à visualiser les différences entre les neuf populations d'une part sur les indices de diversité génique (N_a et H_e) et d'autre part sur les indices affectés par le régime de reproduction (*CG:N* et $\overline{r_D}$). Une Analyse en Composantes Principales a ainsi été réalisée en prenant les populations comme individus et les quatre indices comme variables. Les deux premières Composantes Principales (PC) résument environ 96% de l'information (Figure 6.1). La PC1 est positivement corrélée avec les indices N_a, H_e et *CG:N* et négativement corrélée avec l'indice $\overline{r_D}$ alors que la PC2 est positivement corrélée avec l'indice H_e et négativement corrélé avec l'indice $\overline{r_D}$ (Figure 6.1a). La représentation sur le plan (1,2) a permis d'identifier deux groupes de populations (Figure 6.1b). Le premier groupe, caractérisé par des fortes valeurs de N_a, H_e et *CG:N* et de faibles valeurs de $\overline{r_D}$ comprend les populations asiatiques CH1, CH2, CH3 et TH. A l'inverse, le deuxième groupe caractérisé par de faibles valeurs de N_a, H_e et *CG:N* et de fortes valeurs de $\overline{r_D}$ comprend la population chinoise CH4 et les populations de Colombie (CL), de France (FR), d'Amérique du Nord (USA) et de Madagascar (MD). Les résultats sur les indices *CG:N* et $\overline{r_D}$ soutiennent l'hypothèse de reproduction sexuée dans les populations asiatiques, en particulier dans la population CH1, et l'hypothèse de reproduction asexuée dans les autres populations.

Delmotte *et al.* (2002) ont suggéré que la relation entre la proportion de génotypes multilocus (MLG) observés dans un échantillon et le nombre de loci étudiés était différente entre une population sexuée et une population clonale (Figure 6.2). Dans une population sexuée, peu de loci suffisent à discriminer rapidement le nombre total de MLG, ce qui se traduit par une courbe avec une augmentation rapide puis un plateau. Dans une population clonale, en revanche, les nouveaux MLG ne sont obtenus que par mutation, évènement plus rare que la recombinaison, ce qui a pour conséquence une relation linéaire de faible pente entre le nombre de MLG et le nombre de loci.

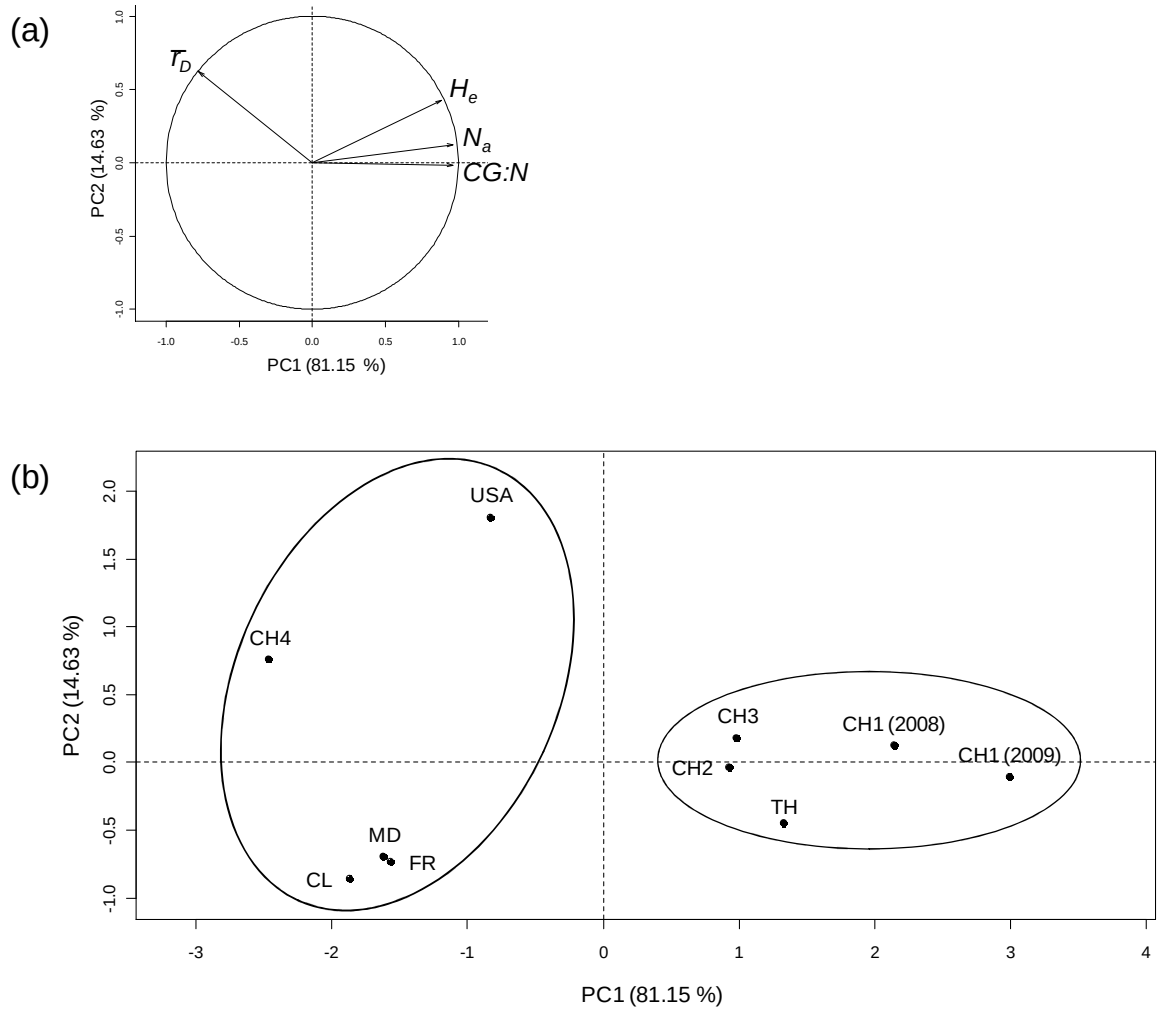

Figure 6.1. Analyse en Composantes Principales (ACP) en prenant les neuf populations comme individus et les indices N_a, H_e, *CG:N* et $\bar{r}_D$ comme variables.
a, Cercle des corrélations. **b,** Projection des individus sur le plan factoriel (1,2). La proportion d'inertie du plan (1,2) est de 95.78%. Tous les individus sont bien représentés ($cos^2 > 1/10^e$).

Dans notre étude, la proportion de MLG (G:N) discriminés en fonction du nombre de loci étudiés a été représentée, pour chacune des populations de l'article 2, en figure supplémentaires. Cette figure a été refaite afin de mettre en évidence les différences entres populations sexuées et populations asexuées (Figure 6.3). Chaque valeur de G:N a été moyennée sur 1000 tirages sans remise parmi les 17 marqueurs microsatellites étudiés. Dans les populations d'Asie où la reproduction sexuée est suspectée (CH2, CH3 et TH) ou confirmée (CH1), la forme de la relation entre nombre de MLG et nombre de loci est compatible avec un régime sexué puisqu'elle montre une augmentation rapide suivie d'un plateau. Dans les populations clonales (CH4, CL, FR, USA et MD), la relation est linéaire avec une pente faible et est compatible avec l'hypothèse de clonalité.

Par ailleurs, pour chacune des neuf populations, des arbres des individus ont été construits à partir de matrices de dissimilarités (Figure 6.4). La population dans laquelle la recombinaison a été montrée dans l'article 1 (CH1) présente, pour chacune des deux années (2008 et 2009), des branches mal résolues, alors que les populations clonales (CH4, CL, FR, USA et MD) montrent des branches en « râteaux ». Les populations TH, CH2 et CH3 montrent une structure intermédiaire, témoignant soit d'une reproduction sexuée contemporaine mais moins intense que dans la population CH1, soit d'évènements de recombinaison ancestraux.

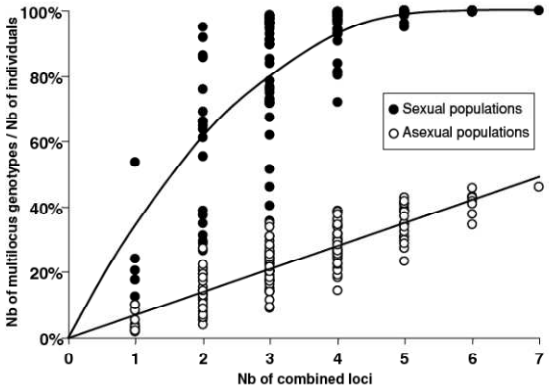

Figure 6.2. Proportion de génotypes multilocus (MLG) discriminés en fonction du nombre de loci étudiés dans des populations de *Rhopalosiphum padi*.
(d'après Delmotte *et al.*, 2002)
La proportion MLG a été calculée comme le nombre MLG divisé par le nombre d'individus échantillonnés. Les populations sexuées et asexuées sont symbolisées par les cercles noirs et blancs respectivement.

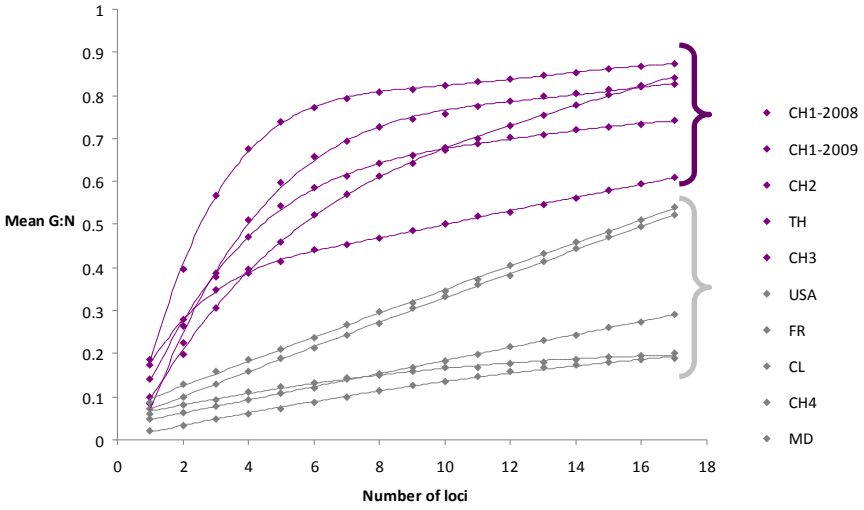

Figure 6.3. Proportion de génotypes multilocus (MLG) discriminés en fonction du nombre de loci étudiés (*G:N*) dans neuf populations de *M. oryzae* (la population CH1 a été échantillonnée deux années consécutives, en 2008 et en 2009).
Les populations chinoises et thaïlandaise dans lesquelles la reproduction sexuée est possible sont symbolisées par les cercles violets et suivent une régression polynomiale d'ordre 4 ou 5. Les populations en dehors de l'Asie et la population chinoise CH4 sont symbolisées par les cercles gris et suivent une régression linéaire (à l'exception de CH4 qui suit une régression polynomiale d'ordre 2, mais se rapprochant d'une régression linéaire).

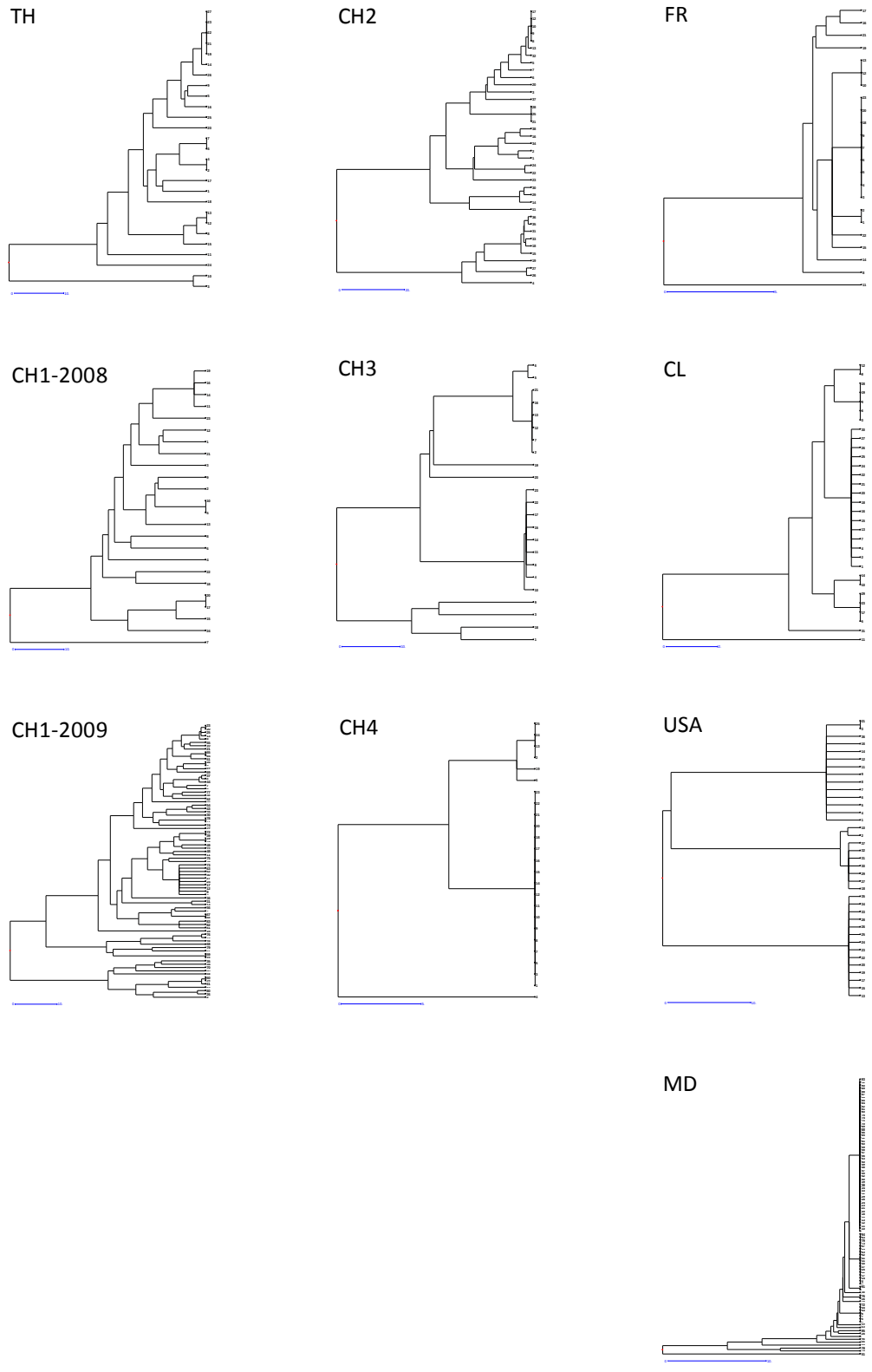

Figure 6.4. Arbres des individus construits sur une matrice de dissimilarités par *Neighbour Joigning* pour neuf populations de *M. oryzae*, à partir de 17 marqueurs microsatellites. La population chinoise CH1 à été échantillonnée deux années consécutives (2008 et 2009) et est donc représentée deux fois.

III. Comment expliquer la perte de reproduction sexuée chez *M. oryzae* ?

1. Protocole expérimental

Annexe 2.

2. Article 3. Asexual reproduction induces rapid and permanent loss of sexual reproduction ability of the fungal rice pathogen, *Magnaporthe oryzae*: results from *in vitro* experimental evolution assays.

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Asexual reproduction induces rapid and permanent loss of sexual reproduction ability of the fungal rice pathogen, *Magnaporthe oryzae*: results from *in vitro* experimental evolution assays.

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Abstract

Background

Sexual reproduction widespread among many eukaryotic microorganisms, and few organisms reproduce exclusively asexually. But, in some species, sexual and asexual reproductions are not exclusive. In many fungi, asexual reproduction alternate with more or less frequent sexual reproduction events. Several fungal species thought to be exclusively clonal were shown to reproduce sexually. Evolutionary shifts from sexuality to asexuality or the reversed have been evidenced. Fungi are thus good models to study why and how sexuality or clonality may be selected for. We worked on *Magnaporthe oryzae*, the Ascomycete fungus responsible for blast disease on rice. This species reproduces mainly asexually *in natura*. Sexual reproduction is possible *in vitro*: it requires two strains of opposite mating types and at least one strain must be female-fertile, that is, able to produce perithecia (the female organs where meiosis takes place). Female-fertile strains are found only in limited areas of Asia where population genetics evidence of contemporary recombination was recently given. We made four Chinese female-fertile strains evolve on artificial medium by transferring conidia from one Petri dish to another every seven days, constituting asexual generations. The aim was to determine if female fertility could be rapidly lost in the absence of sexual reproduction, and if such a loss was under genetic or epigenetic control.

Results

All the strains became female-sterile after 10 to 19 asexual generations. As no monosporic isolation was done during the experiment, the decrease in perithecia production we observed reflected the occurrence and the invasion of a female-sterile mutant in the population of female-fertile individuals. The female-sterile phenotype segregated in the offspring of crosses between female-sterile evolved strains and female-fertile wild-type strains. This segregation was maintained the second generation in backcrosses. Moreover, female-sterile evolved strains were subjected to several stresses, but none of them achieved to restore female fertility. Hence, genetic mechanisms were more likely to explain this loss than epigenetic mechanisms. In competition experiments, we systematically observed that the female-sterile mutant deposited more asexual spores than the corresponding wild-type strain. This advantage is likely to explain why female sterile mutants rapidly invaded during the evolution experiment.

Conclusions

Here we showed for the first time that, in the absence of sexual reproduction, mutants of *M. oryzae* rice strains suppressing female fertility could arise and invade rapidly. This phenotypic alteration, likely caused by mutations, occurred frequently. From these results we hypothesized that female fertility may have been lost rapidly during the dispersion of the fungus from Asia to the rest of the world.

Background

Sexual reproduction is a widespread reproductive mode among many eukaryotic microorganisms, and the proportion of organisms that reproduce exclusively asexually is low (Otto & Lenormand 2002). But, in some species, sexual and asexual reproductions are not exclusive. In fungi, many species reproducing mainly asexually encounter episodes of sexual reproduction (Levin & Bergstrom 2000; Xu 2004b). In addition, recent studies showed that many fungal species thought to be clonal could actually also reproduce sexually (Kück & Pöggeler 2009). For example, Campbell and Carter (Campbell & Carter 2006) have shown recombination in *Cryptococcus neoformans* and *C. gattii*. O'Gorman *et al.* (2009) recently discovered a sexual cycle *in vitro* in *Aspergillus fumigatus.* Seidl *et al.* (2009) identified mating types and induced sexual reproduction for the first time in *Trichoderma reesei*. In total, about 55,000 of the 70,000 known fungal species were identified to reproduce both sexually and asexually (Xu 2004b), and it is likely that sexual reproduction exists in other species but has not been detected yet (Schurko *et al.* 2009). Moreover, in fungi, evolutionary shifts from sexual to asexual reproduction or the reverse, have been evidenced between related species, for example in the genera *Penicillium* (Lopez-Villavicencio *et al.* 2010), *Microsporidia* (Ironside 2007), *Aspergillus* (Geiser *et al.* 1996), or *Candida* (Butler *et al.* 2009). So, fungi are good models to address the question of why and how one or the other mode of reproduction may be selected for.

Among sexually reproducing fungi, reproduction modes are diverse (Billiard *et al.* 2010; Taylor *et al.* 1999). Sexual reproduction is based on the recognition of opposite mating types according to a pheromone/receptor system triggering cell-cell fusion followed by meiosis. Some fungi are heterothallic, opposite mating types being carried by different individuals, implying obligate outcrossing. Conversely, in homothallic fungi both mating types are present in the same genome, and an individual can mate with any other including itself (Heitman 2010). Same-sex mating, that is, an individual being able to reproduce with itself although carrying a single mating type, has also been evidenced in some heterothallic species like *Candida albicans* (Alby *et al.* 2009). Independently from reproduction mode, another key component of sexual reproduction in several fungi is the ability to produce female organs where meiosis takes place. This ability is called female fertility and sexual reproduction is not possible if no female-fertile strain is involved. Besides MAT genes involved in mating types, many loci with quantitative and pleitropic effects, usually involved

in regulation pathways, were shown to contribute to sexual reproduction, and in particular, in female organs differentiation (Xu 2002). Mutations responsible for female-sterile phenotypes have already been reported in such loci in fungi: *Agaricus bisporus* (Durrens 1983; Xu 1995), *Podospora sp*. (Durrens 1983), *Fusarium sp.* (Hornok *et al.* 2007), *Neurospora crassa* (Perkins 1997), *Podospora anserina* (Bernet 1988; Esser & Graw 1980). In addition, epigenetic effects can also be involved in sterile phenotypes (Kelly & Aramayo 2007). Such multifactorial control of sexual reproduction suggests that it could be easily lost by mutations or changes in the expression of one or another of the genes involved (Leslie & Klein 1996). Yet, there are little experimental data supporting the prediction that, in a species where both sexual and asexual reproductions co-exist, female fertility is lost rapidly when populations reproduce only asexually for a time period. Xu (2002) revealed a complete loss of sexual reproduction ability in two strains of *Cryptococcus neoformans* after several asexual generations *in vitro*, and estimated the associated minimum genome-wide mutation rates for each strain (0.0172 and 0.0772). However, whether the causes underlying the apparition of this mutant phenotype were genetic or epigenetic mechanisms was not assessed.

Here we aimed at evaluating experimentally if female fertility could be selected against in the absence of sexual reproduction events. We used the fungal species *Magnaporthe oryzae* for which sexual reproduction ability is well documented. This Ascomycete fungus is responsible for the most important fungal disease on cultivated rice worldwide: blast (Dean *et al.* 2005; Valent 1990).

In *M. oryzae*, the pathogenic cycle observed in the field is an asexual cycle. The rice blast fungus attacks all aerial parts of the plant. Foliar infection is initiated by attachment of asexual spores (conidia) to the rice leaf cuticle (Wilson & Talbot 2009). Conidia produce an appressorium that penetrates host tissue, and form a germ tube into the host cell. Then the fungus produces mycelium and colonizes the host tissue (Wilson & Talbot 2009). After 5 to 7 days, lesions appear and, under moist conditions, conidiophores and conidia are produced outside the plant, starting a new cycle. To date the sexual stage has never been observed in the field. However, some strains from the centre of origin of the fungus (Himalayan Foothills) are able to reproduce sexually *in vitro* and population genetics studies have shown that sexual reproduction still occurs, at least in limited areas in this region (D. Saleh, E. Fournier, and D. Tharreau, personal communication). *M. oryzae* is a heterothallic species. The two mating types, Mat1.1 and Mat1.2, are determined by two idiomorphs located on chromosome seven

(Kanamori *et al.* 2007). Moreover, for sexual reproduction to occur between two strains, at least one of them, regardless of the mating type, must be able to produce perithecia (female-fertile). Female-fertile strains are rare and are almost exclusively found near the putative centre of origin of the fungus (Zeigler 1998). Hence, the species encompasses populations where individuals alternate sexual and asexual reproduction in the centre of origin, and populations with exclusively clonal individuals in the rest of the world. The most parsimonious hypothesis is that *M. oryzae* may have lost the ability to reproduce sexually during its spread from the centre of origin to the rest of the world. If so, there could be a selective advantage to reproduce asexually. *M. oryzae* is thus a well-suited biological model to study how sexual reproduction ability may be lost within the same species. Moreover, the loss of female fertility has already been observed *in vitro* in *M. oryzae* isolates from finger millet (*Eleusine coracana*) (Tharreau *et al.* 1997). However, the genetic mechanisms involved in this shift are still unknown.

Our objective was thus to determine if female fertility could be rapidly lost in rice strains of *M. oryzae* when sexual reproduction is not taking place, and if so, to assess if the loss was under the control of genetic or epigenetic factors. For this purpose, we followed the evolution of female fertility in the absence of sexual reproduction in an *in vitro* experiment. We then generated progenies between evolved female-sterile strains and wild-type strains to study the segregation of the female-sterile phenotype. We also tested if female fertility could be restored by applying several stresses to the evolved female-sterile strains. We studied the putative pleiotropic effects accompanying the loss of female fertility. Finally, we performed competition experiments to test whether female-sterile strains had a fitness advantage.

Methods

Collection and storage of strains

We chose four female-fertile strains collected in 2008 from rice in one population in South China (Table 7.1). In this sample, 19 on 24 strains were female-fertile, and all population genetics indices (genotypic diversity, linkage disequilibrium) were consistent with sexual reproduction (D. Saleh, E. Fournier and D. Tharreau, personal communication).

Strain	Mating type	Reference strains (ref.) for crosses	Replicate	Last culture	Duration of the experiment ("clonal generations"/days)	R^2		a		b		t_{50}		ρ	
						1st ref.	2nd ref.	1st ref.	2nd ref.	1st ref.	2nd ref.	1st ref.	2nd ref.	1st ref.	2nd ref.
S1	MAT1	S3 and S4	A	S1-A10	10/73	0.03	0.01	4.4	4.9	0.02	-0.01	-	-	-0.65	0.69
			B	S1-B20	20/146	0.82	0.53	4.8	5.1	-0.28	-0.15	2.5	4.5	0.56	0.64
S2	MAT1	S3 and S4	A	S2-A20	20/146	0.32	0.32	5.4	5.5	-0.08	-0.08	8.6	8.4	0.38	0.60
			B	S2-B20	20/146	0.85	0.94	5.4	5.6	-0.21	-0.24	3.3	2.9	0.82 *	0.98 *
S3	MAT2	S1 and S2	A	S3-A10	10/73	0.36	0.67	5.5	5.7	-0.14	-0.20	3.7	3.5	0.64	0.90 *
			B	S3-B20	20/146	0.67	0.87	5.6	5.8	-0.19	-0.30	3.7	2.3	0.65	0.82 *
S4	MAT2	S1 and S2	A	S4-A20	20/146	0.46	0.94	5.8	5.4	-0.13	-0.40	5.4	1.7	0.84 *	0.95 *
			B	S4-B20	20/146	0.60	0.99	5.7	5.6	-0.22	-0.46	3.2	1.5	0.89 *	0.85 *

Table 7.1. Strains used for the experiment, mating type, experimental design and parameters of the Poisson regressions adjusted to the data.
Two replicates were performed for each strain (A and B). The name of experimental strains is given as X-R_{AG} with X: name of the original wild-type strain, R: replicate (A or B) and AG: number of "asexual generations". The duration of the experiment is given in number of AG and number of days. The "intercept" (a) and "slope" (b) of the Poisson regressions $y = e^{a+bt}$ (y: number of perithecia produced by the evolved strain, t=number of AG) and the t_{50} are given for each evolved strain with the two reference strains (ref. st.). R^2: Coefficient of determination for each fit. ρ: Pearson's correlation coefficients between female fertility (=number of perithecia produced) and male fertility (=number of perithecia induced) for each strain against each reference strain in each replicate (* indicate values significantly different from 0, test for association between paired samples).

Two of the chosen strains were Mat1.1 (CH999, hereafter named S1, and CH1003, hereafter named S2) and the two others were Mat1.2 (CH997, hereafter named S3, and CH1019 hereafter named S4). All the experiments (strain cultures and sexual crosses, see below) were performed on rice flour agar medium (rice flour 20g; yeast extract 2g; agar 15g; water 1L; and Penicillin G 500,000 UI added after autoclaving 20min at 120°C).

Experimental evolution

We generated two replicates (A and B) of experimental evolution for each of the four strains described above. At the beginning of the experiment, each strain was grown at 25°C on rice flour agar medium in a ∅ 90 mm Petri dish. After seven days, the culture underwent two parallel treatments:

(i) storage at -20°C on dried filter papers as described by Valent *et al.* (1986). No monospore isolation was performed at this step.

(ii) transfer of asexual spores (conidia) to a new ∅ 90 mm Petri dish, to initiate the next culture. It was performed by inverting and stamping the first Petri dish on a new dish.

The procedure was repeated consecutively 10 to 20 times, and we called “asexual generation” (AG) the seven-days period separating two consecutive transfers. An AG encompassed at least 220 mitotic multiplication cycles. This number was estimated by dividing the distance colonized on a petri dish in one AG (2.2 cm) by an average mycelium cell size (100 µm). For each replicate of each strain, we obtained 10 to 20 experimental strains, one by AG. The evolved strains were given the following designation Sx-R_{AG} with Sx: name of the original wild-type strain, R: replicate (A or B), and AG: number of asexual generations. For example, S3-A_5 was the strain evolved from S3 during five AG in replicate A.

At the end of the experiment, fresh cultures of all evolved strains were restarted from the stocks performed at each AG. These cultures were used to evaluate the female fertility of each evolved strain, by testing them against reference strains of known mating types as described below.

Measure of fertility

Sexual reproduction of *M. oryzae* can occur when mycelia from strains of opposite mating types come into contact, if at least one of them is female-fertile. Fertilizing elements are not clearly established in *M. oryzae.* Microconidia were recently described and are suspected to be male elements but their role in sexual reproduction was not demonstrated (Chuma *et al.* 2009; Zhou *et al.* 2011). Conidia do not seem to be involved since crosses between two aconidial mutants produced the complete sexual cycle (Shi & Leung 1995). In *M. oryzae* meiosis is immediately followed by one mitosis to generate four pairs of sister ascospores grouped in the asci. The ascospores are formed within the sexual structures -the perithecia- produced by the female-fertile strain (by the two strains if both are female-fertile).

To determine the female fertility of the evolved strains, we evaluated their ability to produce perithecia when confronted to reference strains. Reference strains are hermaphroditic strains of known mating type producing numerous perithecia when crossed with a strain of opposite mating type (Figure 7.1A, B). Here, the four wild-type strains were used as reference strains in crosses with evolved strains. For the evolved strains from S1 and S2 (Mat1.1), both S3 and S4 (Mat1.2) were used as references. For the evolved strains from S3 and S4 (Mat1.2), both S1 and S2 (Mat1.1) were used as references. These crosses also allowed evaluating male fertility of each evolved strain, that is, the ability to induce the production of perithecia in the reference strains.

Crosses were performed as described by Nottéghem & Silué (1992) on rice flour agar medium in ∅ 90 mm Petri dishes. After two days at 25°C, the cultures were placed under continuous light at 20°C. After 21 days, the production of perithecia was observed. Two rows of perithecia between a tested strain and a reference strain meant that both strains were male-fertile and female-fertile (Figure 7.1C). The observation of a single row meant that only the reference strain produced perithecia, and that the tested strain was female-sterile, but male-fertile (Figure 7.1D). The absence of perithecia meant that the tested strain was both female-sterile and male-sterile. The crosses between each wild-type strain and the reference strains of opposite mating type were used as positive controls compared to evolved strains. For each strain and experiment, evolved strains were collected at different AG and the number of perithecia they produce was estimated on each line by counting the perithecia in

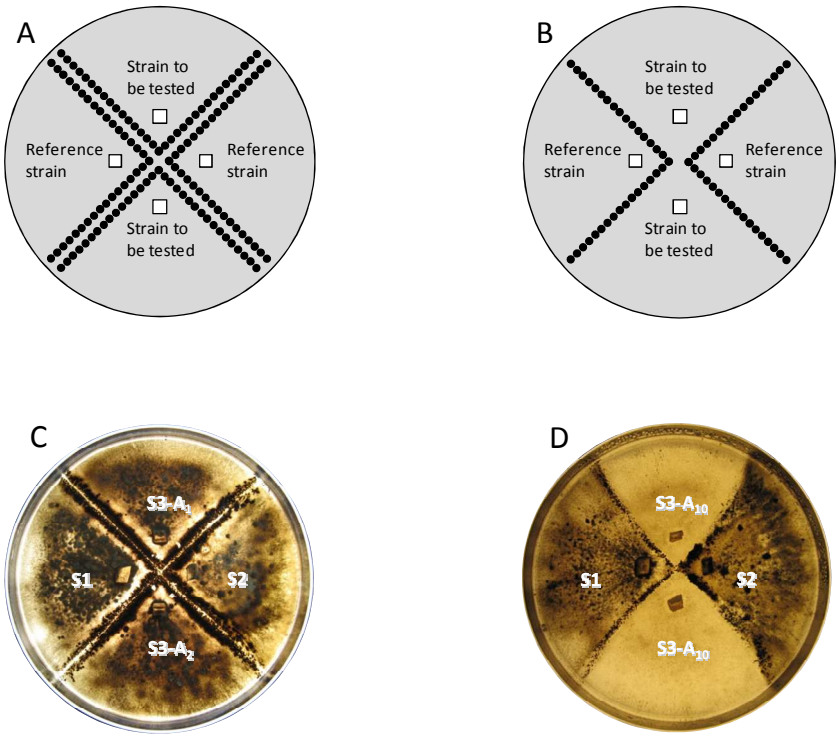

Figure 7.1. *Magnaporthe oryzae* sexual crosses on rice flour agar medium.
Design of the crosses (A) and schematic representation of results (B). A. Two lines of perithecia are produced between each tested strain and each reference strain: the tested strains are female-fertile. B. A single line of perithecia is produced between each tested strain and each reference strain: the tested strains are female-sterile. C and D: pictures of crosses with evolved strains derived from S3 at three different asexual generations (1, 2 and 10) with the two reference strains S1 and S2. C. Tests on S3-A_1 and S3-A_2: both strains are female-fertile. D. Tests on S3-A_{10} (same strain tested twice): the strain is female-sterile.

	Proportion of female-fertile replicates before stress				Proportion of female-fertile replicates after stress			
stress	S1	S3	S1-B_{12}-	S1-A_{10}	S1	S3	S1-B_{12}	S3-A_{10}
-80℃ (1h)	5/5	5/5	0/5	0/5	5/5	5/5	0/5	0/5
-80℃ (24h)	5/5	5/5	0/5	0/5	5/5	5/5	0/5	0/5
sonication	5/5	5/5	0/5	0/5	5/5	5/5	0/5	0/5
monosporic isolation	1/1	1/1	0/1	0/1	12/12	12/12	0/12	0/10
growth on plant	1/1	1/1	0/1	0/1	7/10	10/10	0/08	0/10

Table 7.2. Stresses performed on female-sterile strains S1-B_{12} and S3-A_{10} and their respective corresponding wild-type strains S1and S3.
The treatments were: one hour at -80°C, 24 hours at -80°C, mycelium mashing using ultrasounds, monosporic isolation and growth on plants. The ratios number of replicates where the strain was female-fertile/number of replicates where strains were female-sterile are given before and after the different treatments.

three 3x3mm squares evenly distributed along the corresponding perithecia line. When no perithecia were detected in the squares, we verified that no perithecia were formed at all on the Petri dish.

Obtaining progeny for genetic studies

We isolated progeny from two crosses between a wild-type strain and an evolved strain that had lost female fertility: S3-A_{10} was crossed with S1 (cross #126) and S1-B_{12} was crossed with S3 (cross #127). For ascospore isolation, 19-21 days after crossing (see above), mature perithecia were placed on water-agar medium (40g bacto-agar in 1L distilled water) and opened with a scalpel to release asci. The asci were separated with a fine glass needle, and, after 20-30 min, were gently crushed to release ascospores. These latter were separated with a fine glass needle and incubated at 25°C for 24h. Germinated ascospores were then transferred to rice flour agar medium and stored as previously described. We kept only one germinated ascospore per ascus, ensuring that each progeny comes from an independent meiotic event and avoiding collection of sister ascospores.

Reprogramming of gene expression in female-sterile evolved strains

In an attempt to reverse potential epigenetic effects, we applied to female-sterile evolved strains different treatments that are known or very likely to cause reprogramming of gene expression (Silar *et al.* 1999; Lalucque *et al.* 2001). We tested cold temperature and mycelium fragmentation on S3-A_{10} and S1-B_{12} and on the corresponding wild-type strains S3 and S1 (Table 7.2). These treatments were harmful enough to be considered as stresses without killing the mycelium or the conidia. On the same strains, we also performed two treatments that were shown to produce reprogramming of gene expression: monosporic isolation and growth on plant (Table 2).

For each treatment, the mycelium or spores were collected before and after the treatment and used to evaluate female fertility according to the method describe above. For the extreme temperature stress, strains were grown on rice flour medium for one week and

then placed at -80°C: half for 1h and half for 24h. For the mycelium fragmentation stress, strains were grown on rice flour medium for one week and the surface of the medium was watered, scrubbed, and the harvested mycelium was placed in tubes. Ultrasounds were applied for 30 seconds on the suspension to break the mycelium and a drop of the suspension was placed on rice flour medium for crosses. For monosporic isolation, single conidia were collected and spread on Bacto-agar medium (40g/l) using a fine glass needle. After 24h, 10 germinated spores were collected and used for crosses with reference strains. For in planta growth, strains were inoculated on rice plants as described above. Infected leaves were collected and placed for lesion sporulation on a moist filter paper for 24h. Conidia were then collected using a fine glass needle and isolated as described above.

Measure of asexual sporulation

In vitro asexual sporulation was estimated for seven female-sterile evolved strains (S1-$B_{12,}$ S2-A_{19}, S2-$B_{12,}$ S3-A_{10}, S3-B_{15}, S4-A_{19}, and S4-B_{10}), and for the corresponding wild-type strains (S1, S2, S3, S4). Two replicates were performed per strain. In an additional experiment, five replicates were realized for S1-B_{12}, S3-A_{10}, S1, and S3. After seven days of growth on rice flour agar medium, conidia were collected by watering the plate and scrubbing the surface of the medium. The concentration was estimated by counting spores under the microscope with a hemocytometer. The mycelial surface on the Petri dish, which reflects growth speed, was estimated by image analysis using the LEICA APPLICATION SUITE 3.7.0 software. We calculated the sporulation rates by dividing the spore counts by this mycelial surface. The capacity of conidia to germinate and to form appressoria (the organ essential to host tissue penetration) was measured for S1-$B_{12,}$ S3-A_{10}, S1, and S3. Calibrated conidia suspensions were deposited on glass slides in a Petri dish with humid filter paper and the proportion of germinating conidia and of conidia forming an appressorium were observed after 24h at 25°C. The sporulation rates were also calculated on 12 and 15 progenies from cross #126 and cross #127 respectively (two replicates for each progeny). In this experiment, growth area was evaluated by measuring the colony diameter with a ruler.

In planta asexual sporulation was also evaluated for, S1-B_{12}, S3-$A_{10,}$ S1, and S3. Four trays of c.a. 150 rice plants (variety Maratelli) were grown for four weeks in the greenhouse

and inoculated with conidia suspensions (20ml of a 25,000 spores.ml^{-1} suspension with 1% gelatin) of each strain independently as previously described (Berruyer *et al.* 2003). After one week, the number and the surface of lesions were estimated on 15 leaves for each strain. On each of the 15 leaves, a single lesion was collected. Lesions were pooled by three in tubes with 1ml water and Tween (five replicates), and mixed thoroughly. Conidia concentrations were estimated using a hemocytometer and the sporulation rate was calculated by dividing the spore counts by the surface of the lesions.

Competition experiments between female-sterile and female-fertile strains

We also performed quantitative measures of fitness in competition between female-sterile evolved strains and their corresponding female-fertile wild-type strains. We let the wild-type and evolved strains grow together during one AG. Among the conidia that germinate after transfer to a new Petri dish, we estimated the proportion of female-fertile and female-sterile. This experiment was done between female-sterile evolved strains S1-B_{20}, S2-A_{20}, S3-B_{20} and S4-A_{20} and their respective female-fertile wild-type strains S1, S2, S3 and S4. We prepared eight suspensions of conidia (5000 spores per ml) for each of these eight strains. We also prepared four suspensions (5000 spores per ml) in which evolved strains were mixed with their corresponding wild strain (S1/S1-B_{20}, S2/S2-A_{20}, S3/S3-B_{20} and S4/S4-B_{20}). For each of the 12 strains or mixture, 40µl of suspensions were deposited on a Petri dish (2x20µl for mixtures). Two replicates were performed for each strain or mixture. After seven days of growth at 25°C, i.e. one AG, conidia were transferred to a new Petri dish as described earlier. After 24h at 25°C, we first estimated the number of conidia transferred by counting them in three ∅ 3 mm disks. For female-fertile strains grown alone, the results thereby obtained allowed us to estimate the minimum number of conidia transferred at the end of an AG during the evolution experiment described above. For that, we simply multiplied the average number of spores counted on the three disks by the surface of a Petri dish (6359 mm^2) corrected by the surface of disk (7 mm^2). We also recorded the number of germinated conidia, and. averaged the number of germinated and non-germinated conidia over the three counted disks and over the two replicates. For mixtures of female-sterile and wild-type strains only, we then collected 20 germinated conidia. Their female-fertile or female-sterile phenotype was determined by

crossing them with the appropriate reference strains. This measure gave an estimate of the proportion of female-fertile germinated conidia (i.e. produced by the wild-type strain) *versus* female-sterile germinated conidia (i.e. produced by the evolved strain) that had been transferred. The values were averaged over the two replicates.

Statistical analyses

Statistical analyses were performed using the R software. For each evolved strain the numbers of perithecia (y) they produced (female fertility) and they induced to the reference strains (male fertility) in confrontation with the two reference strains, were plotted as a function of the number of clonal generations (t). We fitted Poisson regressions ($y = e^{a+bt}$) on female fertility and male fertility rather than comparing with the initial values. This method was best suited to take into account the high fluctuations in perithecia production in the first three AG. The number of AG until which the evolved strains produced half of the initial number of perithecia (t_{50}) was estimated on each fit for female fertility curves. The putative effects of mating type (Mat1 and Mat2), wild-type strain (S1, S2, S3 and S4), replicates (A and B), and reference strain on t_{50} were tested using an analysis of variance (ANOVA). *In vitro* and *in planta* differences of asexual sporulation and differences of mycelial surface on Petri dish between the evolved strains S1-B_{12} and S3-A_{10} and the corresponding wild-type strains S1 and S3, were tested by Kruskall-Wallis non parametric tests. Departure from expected segregations or ratios were tested by a χ^2 test.

Results

Loss of female fertility

The aim of the experiment was to study the evolution of female fertility during several generations of asexual reproduction. For this, crosses of the strains at different AG were performed to estimate the proportion of perithecia they produced.

As expected, the level of female fertility observed at t_0 for each wild-type strains differed according to the reference strain used for crosses. For example, the number of perithecia produced by S3 was two times higher when confronted to S1 than when confronted to S2. This may reflect specificities in the interactions between some strains.

The variation of female fertility (number of perithecia produced by the evolved strains) and of male fertility (number of perithecia induced by the evolved strains but produced by the reference strains) with AG, monitored for S1, S2, S3 and S4 (two replicates) is presented in Figure 7.2. The decreases in female fertility and male fertility both fitted Poisson distributions ($y = e^{a+bt}$). For female-fertility, the "intercept" (a), the "slope" (b) and the time until the number of perithecia was reduced by two (t_{50}) are given in Table 1.a complete loss of female fertility in all strains and replicates, except for replicate A of strain S1. For this evolved strain, although a slight decrease was observed in the first AG, female fertility increased again and never reached zero. The experiment lasted only 10 AG for this replicate (vs 20 AG for replicate B), and this duration was perhaps not sufficient for this strain to evolve towards female sterility. t_{50} reflects the speed at which female fertility was lost, so it was not calculated for S1-A. For S1-B, the number of perithecia became null after 12 AG of experimental evolution. For all the other strains, female fertility was completely lost in 10 to 19 AG in both replicates. The time until complete loss of female fertility varied between strains and between replicates. In S2, female fertility was lost in 19 AG in replicate A and 12 AG in replicate B. In S3 female fertility was lost in 10 AG for replicate A and in 15 AG for replicate B. In S4, female fertility was lost in 19 AG in replicate A and 10 AG in replicate B. An ANOVA revealed significant effects of mating type and replicate on t_{50} (Table 7.3). So female fertility was almost always lost for each strain but the speed of this loss was not the same for both replicates of the same strain, and also significantly differed according to the mating type of the strain.

Since no single spore isolation was realized in the time course of the experiment, intermediate AG are expected to be mixtures of female-fertile and female-sterile individuals. To verify this, 10 monosporic strains were isolated at the fifth AG of experiment A for each

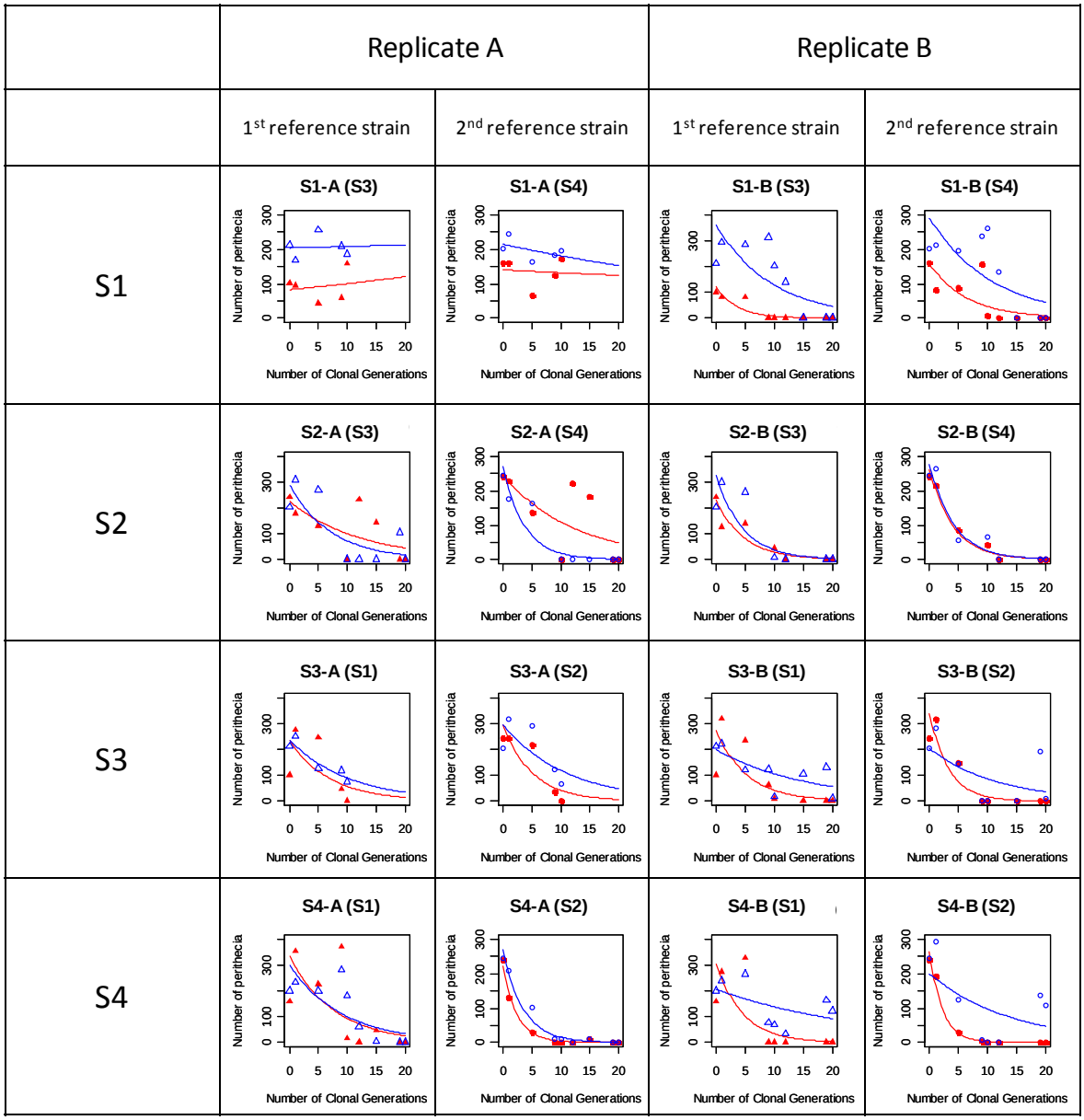

Figure 7.2. Estimation of the number of perithecia produced during crosses between the strains after several AG.

Perithecia were counted on 9 mm^2 squares at three different positions along the confrontation line. Filled red points: number of perithecia produced by the evolved strain (female fertility). Empty blue points: number of perithecia produced by the reference strain,that is, induced by the evolved strains (male fertility). Triangles: first reference strain; Circles: second reference strain. The name of the reference strain used for the crosses is given into brackets. The curves represent the Poisson regressions fitted on data: $y = e^{a+bt}$.

Source of variation	df	Sum of squares	Mean Square	F values	P values	
Mating type	1	12.4	12.4	12.5	0.017	*
Wild-type strain	2	7.4	3.7	3.7	0.103	
Replicate	3	31.1	10.4	10.4	0.014	*
Reference strain	2	6.3	3.1	3.1	0.130	
Residuals	5	5.0	1.0			

Table 7.3. ANOVA analyzing the differences of t50 for loss of female fertility according to mating types, strains, replicates and reference strains used for crosses.
df: degrees of freedom.

evolved strain. These monosporic strains were then tested for female fertility. The number of female-sterile monosporic strains was 3, 5, 1, and 2 for S1-A_5, S2-A_5 S3-A_5, and S4-A_5, respectively. Hence, since intermediate AG are mixtures of female-fertile and female-sterile individuals, the quantity of perithecia produced measured over the different AG likely reflects the proportion of female-fertile strains in the mixture. This result also confirms that female-sterile strains appeared very early in the experiment (before 5^{th} AG).

For two strains, another phenotypic modification was observed during the experiment. In S2 in both replicates at the 9^{th} AG, perithecia were formed all over the plate and not only at the confrontation line between the different mycelia. At the 10^{th} AG, this phenotype disappeared in both replicates but reappeared in replicate A between the 12^{th} and the 15^{th} AG (corresponding to a peak in number of perithecia in Figure 7.2). The same phenotype was observed in replicate A of S4 at the 15^{th} AG. No ascospores were found in these perithecia. This phenotype may correspond to the self-formation of sterile perithecia which has already been observed in finger millet strains of *M. oryzae* after subculturing (Tharreau *et al.* 1997). At the end of the experiment these strains became female-sterile and male-sterile. We verified that the fitted Poisson regressions remained unchanged when self-formed perithecia were removed from the datasets. With this correction, the regressions had higher R^2 values but the parameters values were only slightly different (data not shown).

Decrease in male fertility

Male fertility was also affected during the experiment. Poisson regressions were also fitted on the number of perithecia produced by the reference strains when crossed with the evolved strains. At the end of the experiment, male fertility was completely lost for about half of the replicates: S1-B, S2 (replicates A and B), S3-B and S4-A. To test whether modifications in female fertility and male fertility over time were correlated, we used Pearson's correlation tests to compare the number of perithecia produced *versus* induced by the evolved strains at different AG. Positive significant correlations were found, but neither for all strains nor for all replicates (Table 7.1). Male fertility and female fertility were positively correlated for S4 for both replicates A and B in crosses with both references. A positive significant correlation was also found for S2, but only for replicate B in crosses with

both references. The correlation was also positively significant for S3 for both replicates A and B but only in crosses with reference strain S2. There was no significant correlation between male fertility and female fertility for S1 for replicates A and B in crosses with both references. So, correlation between male fertility and female fertility over time could not be generalized.

Have the evolved female-sterile phenotypes an epigenetic or genetic base ?

Since the loss of female fertility was frequent and rapid in our experiment, we first suspected an epigenetic control. Phenotypic modifications controlled by epigenetic mechanisms can be reversed by reprogramming of gene expression following stresses or developmental processes (Silar *et al.* 1999). We thus addressed the question of an epigenetic control of female sterility by testing whether several stresses (cold, mycelium fragmentation) and developmental processes (formation of conidia, host infection) applied to the female-sterile evolved strains S1-B_{12} and S3-A_{10} could restore the wild-type female-fertile phenotype. As control, we applied the same stresses to the wild-type strains S1 and S3 and tested their female fertility after treatments. Whatever the treatment imposed, the two wild-type strains remained female-fertile, confirming that the chosen treatments were not likely to alter female fertility. None of the treatments or cellular events tested (including meiosis, see below) reversed the female-sterile phenotype of the evolved strains S1-B_{12} and S3-A_{10} (Table 7.2). Although they do not rule out the hypothesis of an epigenetic control, these results support the hypothesis of a genetic origin of the experimental loss of female fertility.

We then tested the genetic hypothesis by performing crosses between female-sterile evolved strains and wild-type strains, in order to determine whether the evolved phenotype segregated in the offspring and to determine the number of genes involved in the loss of female fertility (Table 7.4). As expected, offspring of the control cross between the two wild-type female-fertile strains S1 and S3 (cross #125) were all female-fertile. In the offspring of the cross between S1 and S3-A_{10} (cross #126), the ratio of female-fertile:female-sterile strains was significantly different from 1:1 (one gene) but not significantly different from 1:3 (two genes; χ^2=1.13, P=0.29, df=1). Backcrosses were also performed between a Mat1.1 female-sterile progeny (126/0/4) and the wild-type strain S3 (cross #130) and between a Mat1.2

Cross number	Parental strains		Parental phenotypes		Observed segregation				Mat1:Mat2		f:m		
					Mat1-f	Mat2-f	Mat1-m	Mat2-m	Observed	Chi² and P (1:1)	Observed	Chi² and P (1:1)	Chi² and P (1:3)
	S1	S3	Mat1-f	Mat2-f	21	18	0	0	21:18	0.23 (0.631)	39:00		
126	S1	S3-A10	Mat1-f	Mat2-m	**6**	3	15	**10**	21:13	1.88 (0.170)	12:24	7.53 (0.006)	**0.04 (0.998)**
130	S3	126/0/04	Mat2-f	Mat1-m	7	**2**	**11**	15	18:17	0.03 (0.866)	09:26	8.26 (0.004)	**0.01 (0.922)**
133	S1	126/0/35	Mat1-f	Mat2-m	**3**	1	2	**6**	05:07	0.33 (0.564)	04:08	**1.33 (0.248)**	**0.40 (0.505)**
127	S3	S1-B12	Mat2-f	Mat1-m	5	**2**	**15**	15	20:17	0.24 (0.622)	07:30	14.3 (0.000)	**0.73 (0.866)**
131	S1	127/0/25	Mat1-f	Mat2-m	**13**	18	6	**6**	19:24	0.58 (0.446)	31:12	8.40 (0.004)	50.9 (0.000)
132	S3	127/0/28	Mat2-f	Mat1-m	4	**6**	**18**	11	22:17	0.64 (0.423)	10:29	9.26 (0.002)	**0.01 (0.926)**

Table 7.4. Segregation of mating type and female fertility in the progeny of crosses between female-sterile evolved strains S3-A_{10} and S1-B_{12} and wild-type strains S1 and S3, and in backcrosses progeny.

f: female-fertile, m: female-sterile (mutant). The control cross is the cross between the two wild-type strains S1 and S3 (first line).

female-sterile progeny (126/0/35) and the wild-type strain S1 (cross #133). In the offspring of cross #130, the ratio was also not significantly different from 1:3 (χ^2=0.01, P=0.92, df=1). In the offspring of cross #133, the observed ratio did not significantly depart neither from 1:1 nor from 1:3 (χ^2=1.33, P=0.25, df=1, and χ^2=0.40, P=0.51, df=1, respectively). These results suggest that the loss of female fertility in S1-A_{10} is controlled by two independent genes. However the data could also support the hypothesis of one gene with segregation distortion. In the offspring from the cross between S1-B_{12} and S3 (cross #127), the ratio of female-fertile:female-sterile strains was significantly different from 1:1 but not significantly different from 1:3 (χ^2=0.30, P=0.80, df=1). Backcrosses were also performed between a Mat1.2 female-sterile progeny (127/0/25) and the wild-type strain S1 (cross #131) and between a Mat1.1 female-sterile progeny (127/0/28) and the wild-type strain S3 (cross #132). The ratio was not significantly different from 1:3 in cross #132 (χ^2=0.01, P=0.93, df=1). In cross #131, the observed ratio departed from 1:1 (χ^2=8.40, P=0.004, df=1) and from 1:3 (χ^2=50.9, P<0.001, df=1), but it was not significantly different from 3:1 (χ^2=0.19, P=0.66, df=1). Hence, although results of cross #127 and backcross #132 again suggest that the loss of female fertility in S1-B_{12} is controlled by two independent genes, the segregation observed in backcross #131 rather supports a mechanism involving a single distorter gene. Additional crosses are needed to disentangle between the two possible explanations. Nevertheless, for the two analyzed mutants, the segregations of female sterility in the offspring of the first generation cross and in backcrosses support a genetic control of sterility rather than an epigenetic control.

The identity between genes responsible for female sterility in the different mutants could not be tested by classical allelism tests since these mutants were sterile and crosses between them were therefore impossible to perform.

Fitness comparisons between evolved and wild-type strains

Since in our experiments female-sterile mutants replaced rapidly female-fertile strains, we reasoned that the mutants must have a fitness advantage. Fitness describes the ability to both survive and reproduce, and is equal to the average contribution to the gene pool of the next generation that is made by an average individual of the specified genotype or phenotype. In fungi several traits can be related to fitness (Pringle & Taylor 2002). In our experiment, we focused on traits related to asexual multiplication, since it was the unique reproductive mode

considered. Moreover, in fungi, several mutations altering sexual reproduction have been shown to also modify several aspects of the life cycle, and especially asexual reproduction (Hill & Otto 2007). We used different approaches to address this point.

Fitness traits measured in female-fertile wild-type and female-sterile evolved strains grown separately.

The first approach was to compare several traits involved in fitness between wild-type and evolved strains grown separately. We focused on three particular traits related to asexual reproduction: the speed of vegetative growth, the rate of asexual sporulation (*in vitro* and *in planta*), and the number of asexual conidia transferred *in vitro* at the end of an AG.

Mycelial growth speed was measured as the mycelium surface that had colonized the Petri dish after seven days. It was significantly lower in S3-A_{10} than in S3 (Kruskall-Wallis χ^2=6.81, P=0.009, df=1), but it was not significantly different between S1-A_{12} and S1 (Kruskall-Wallis χ^2=1.84, P=0.17, df=1).

We then compared the *in vitro* production of conidia between evolved strains S1-B_{12} and S3-A_{10} and their corresponding wild-type strains S1 and S3 (Figure 7.3A). In the evolved strain S3-A_{10}, the *in vitro* asexual sporulation was significantly divided by four compared to the corresponding wild-type strain S3 (Kruskall-Wallis χ^2=6.82, P=0.009, df=1). This held also for the evolved strain S1-B_{12} for which asexual sporulation was divided by two compared to the corresponding wild-type strain S1 (Kruskall-Wallis χ^2=4.81, P=0.028, df=1). For the other evolved strains that had lost female fertility (data not shown), although only one or two replicates were done, we also observed a trend towards a reduction of asexual sporulation (five times lower for S3-B_{15} compared to S3, 1.5 times lower for S2-A_{19} and two times lower for S2-B_{12} compared to S2, and 1.5 times lower for S4-B_{10} compared to S4). This trend was not observed for S4-A. As a whole, these results show that, at least *in vitro*, female-sterile mutants either are unaffected, or are altered in their vegetative growth and in their capacity to produce asexual spores, as compared to their corresponding female-fertile wild-type strains. In progenies of cross #126, we found no significant differences in asexual sporulation between the 8 female-fertile and the A6 female-sterile strains (Kruskall-Wallis χ^2=1.82, P=0.18, df=1). We did not either found differences in asexual sporulation between the 11

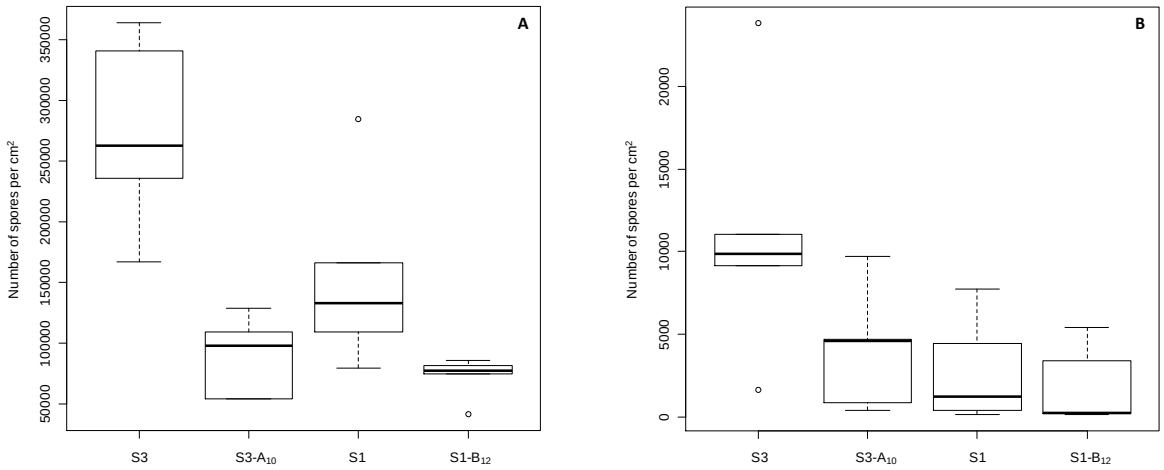

Figure 7.3. Number of conidia per cm^2 produced by the evolved strains S1-B_{12} and S3-A_{10} and the corresponding wild-type strains S1 and S3, respectively.
Conidia production was measured in vitro (A) and on rice plants (B).

female-fertile and the 18 female-sterile strains in progenies of cross #127 (Kruskall-Wallis χ^2=0.01, P=0.93, df=1). So, the genetic mechanisms implied in female-sterility in evolved strains were independent from the genetic mechanisms implied in reduced asexual sporulation.

Previous studies on mutants altered in their sporulation showed reversed results for sporulation between measures *in vitro* and *in planta* (Tharreau *et al.* 1997). We thus compared the *in planta* asexual sporulation between S3-A_{10} and its wild-type strain S3, and between S1-B_{12} and its wild-type strain S1. We also counted the number of lesions and measured their size seven days after plant inoculation. Lesion size was not significantly different, neither between S3 and S3-A_{10} nor between S1 and S1-B_{12} (respectively P=0.084 and P=1, df=1). The number of lesions was significantly lower in S3-A_{10} than in S3 (38% reduction, one-way ANOVA, P=0.027, df=1). Although also reduced by 36%, it was not significantly different between S1 and S1-B_{12} (one-way ANOVA, P=0.051, df=1). Such reduction may be explained by a reduction in conidia germination or appressorium formation. But conidia germination and appressorium formation *in vitro* were not significantly different, neither between S1 and S1-B_{12} nor between S3 and S3-A_{10} (data not shown). For any of the pairs compared, no significant differences in asexual sporulation was observed *in planta* (S3-A_{10} / S3: Kruskall-Wallis χ^2=3.15, P=0.076, df=1 ; S1-B_{12} / S1: Kruskall-Wallis χ^2=0.53, P=0.464, df=1). (Figure 7.3B). So, female-sterile evolved strains S3-A_{10} and S1-B_{12} were altered in their capacity to sporulate on artificial medium but not *in planta*. These results suggest that, although not affected in their asexual sporulation capacity *in planta*, female-sterile evolved strains S3-A_{10} and S1-B_{12} may have a reduced capacity to infect rice plants. However, the results on sporulation obtained *in vitro* or *in planta* cannot explain why female-sterile mutants replaced female-fertile strains in our *in vitro* experiments.

Finally, we tested whether the number of viable conidia (i.e. able to germinate) transferred between each AG could be higher for female-sterile strains compared to female-fertile strains (Figure 7.4). There was no significant difference in the conidia germination rate between wild-type and mutant strains (99.2 to 100%, data not shown). However, the number of viable conidia transferred was higher for the female-sterile evolved strains than for their respective female-fertile wild-type strains. More precisely, there were three times more viable conidia transferred in the female-sterile S1-B_{20} cultures (21.5 / mm² in average) than in the wild-type S1 cultures (8.3 / mm^2 in average). There were about ten times more viable conidia

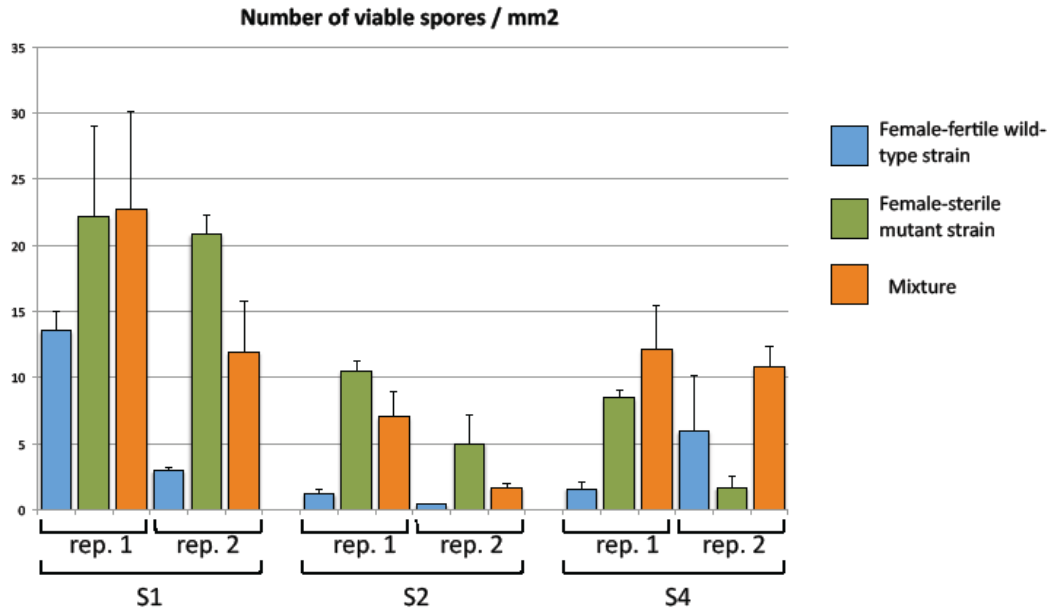

Figure 7.4. Number of conidia estimated on ∅ 3 mm circles that have germinated after transfer from one Petri dish to another. Blue bars: female-fertile wild-type strains (S1, S2 ad S4). Green bars: female-sterile evolved strains (S1-B_{20}, S2-A_{20} and S4-B_{20}). Orange bars: mixture between evolved and wild strains (S1/S1-B_{20}, S2/S2-A_{20} and S4/S4-B_{20}).

transferred in the female-sterile S2-A_{20} cultures (7.7 / mm^2 in average) than in the female-sterile S2 culture (0.8 / mm^2 in average). The number of viable conidia transferred was similar in the female-sterile S4-B_{20} cultures (5 / mm^2 in average) and in the wild-type S4 cultures (3.7 / mm^2 in average). So, for two out of three pairs tested female-sterile evolved strains transferred more conidia than their respective female-fertile wild strains. This result may explain why female-sterile strains invaded the evolved strains.

Fitness traits measured in female-fertile wild-type and female-fertile evolved strains in competition.

To verify if the fitness advantage due to a higher efficiency of conidia transfer also acted in competition, we measured this trait in competition experiments during which the selective treatment was identical to the one performed during the evolution experiment. According to Elena & Lenski (2003), competition experiments between mutants and wild-type phenotypes issued from experimental evolution essays are the most appropriate to evaluate comparative fitness. In our protocol, there is no mean to distinguish between wild-type and mutant strains when they are grown together, either as regards mycelium growth or as regards sporulation rate. On the contrary, the number of viable conidia transferred from each strain at the end of an AG was measurable since, after being transferred, germinating conidia could be isolated and crossed against appropriate reference strains in order to determine their fertility phenotype, hence their parental origin. We proceeded as follows. S1-B_{20} was mixed with S1, S2-A_{20} with S2, S3 with S3-A_{20}, and S4-B_{20} with S4. Single cultures of wild-type and mutant strains performed in the same conditions (see previous section) were used as controls. The competition between S3-B_{20} and S3 was not performed because of experimental problems. After one week, conidia were transferred to a new dish and counted as described above. Again, no significant variation in conidia germination rate was observed (98.6 to 99.8%; data not shown). To determine the ratio of female-sterile and female-fertile strains in these transferred conidia, 20 germinated conidia per mixture were isolated. The derived strains were then tested for female fertility by crossing with testers. For mixture S1 + S1-B_{20} the observed ratio (55%) was not significantly different from the expected 50% ratio (Table 7.5). For mixtures S2 + S2-A_{20} and S3 + S3-$B_{20,}$ 97.5% of the strains recovered were female-sterile. For mixture S4 + S4-$A_{20,}$ 70.0% of the strains recovered were female-sterile, a ratio significantly superior to the 50% ratio expected. These results show that in competition

Mixture	Nb of female-sterile strains recovered over 20 strains		Average % of female-fertile strains recovered	Comparison with expected 50% ratio	Expected % based on conidia transfer evaluation in single culture
	Rep1	Rep2			
S1 + S1B20	10	12	55	NS (khi²=0.4, P=0.522)	72.1
S2 + S2A20	19	20	97.5	S (khi²=36.1, P=0.000)	90.6
S3 + S3B20	19	20	97.5	S (khi²=36.1, P=0.000)	nd
S4 + S4A20	13	15	70	S (khi²=6.4, P=0.011)	42.5

Table 7.5. Ratio of female-sterile and female-fertile strains transferred in one asexual generation from mixtures of female-sterile and female-fertile strains.

the female-sterile mutants of S2, S3 and S4 transferred conidia more efficiently that the female-fertile wild-type strains they derive from.

Discussion

In the present study we demonstrated for the first time that female fertility can be rapidly lost in *in vitro* conditions in *M. oryzae* rice strains grown asexually. All strains we tested experienced a loss of female fertility for at least one replicate. Our results also support the idea that this loss was likely to have a genetic (mutational) rather than an epigenetic origin. The loss of fecundity in laboratory conditions has already been documented in other fungal species such as *Blastomyces dermatidis* and *Histoplasma capsulatum* (Heitman 2010). The loss of female fertility has even been observed in a *M. oryzae* strain from finger millet (Tharreau *et al.* 1997). In this latest study, the female-sterile phentoype was controlled by a single gene. In our experiment, the female-sterile phenotype that appeared during the experiment segregated in the first generation crosses as well as in the backcrosses. This result supports that the loss of female fertility is likely due to genetic mechanisms, either mutations, gene deletion, or major chromosomic rearrangements (Xu 2002), rather that epigenetic mechanisms. Although offspring sample size was not very important here, it was enough to detect segregation. We were expecting a 1:1 segregation of female-fertile:female-sterile strains in the offspring, indicative of the involvement of a single gene. The 1:3 ratio observed in most crosses, suggested the involvement of two genes. However, independent mutations in two unlinked genes seem unlikely. It is even more unlikely that such a phenomenon would have occurred two times, once in strain S1-B_{12} and once in strain S3-A_{10}. Thus, the hypothesis of mutations in a single gene and segregation distortion of this gene is more likely. Additional crosses would be necessary to distinguish between these two hypotheses. Segregation distortion is documented in lots of Eukaryotes and some segregation distorters are known to bias sex-ratio (Kozielska *et al.* 2010; Taylor & Ingvarsson 2003). Theoretical studies suggest that it may play an important role in the evolution of sexual reproduction acting for instance on sex determination (Taylor *et al.* 1999; Lalucque *et al.* 2001; Taylor & Ingvarsson 2003). To determine if the same locus was affected for all strains and all replicates, a suited method would be to perform allelism tests. However, crosses cannot be performed between two

female-sterile strains. Genetic mapping of the mutations would allow comparing genes positions and determining if different loci are involved. Another possibility is to perform complementation experiments by smashing the mycelia of two female-sterile evolved strains together to restore female fertility. We performed this experiment with two strains after isolation of a female-sterile single spore (S2-A_5 and S4-A_5), but did not achieve to restore female fertility until now. A perspective of this work is to perform complementation experiments on all combinations of pairs of female-sterile evolved strains to test the hypothesis of several mutations on different loci.

The demonstration of the rapid loss of such an important life-history trait as female fertility in *M. oryzae* rice strains is new. In an experiment on *M. oryzae* on artificial medium and on plant during ten generations, Park *et al.* (2010) did not find any change in pathogenicity and genome sequences of avirulence genes. They concluded that the genome was highly stable. However, each generation consisted of the transfer of only one or two conidia after two weeks of growth. So, even if mutants appeared at normal rate, the sample size in Park's experiment was not enough to detect them. On the contrary, in our experiment we observed a progressive reduction in perithecia production until complete female sterility. This result is explained by the fact that on average 27000±24000 conidia -constituting a population- were transferred from a Petri dish to the other at each generation. So, the process we observed was the occurrence and the colonization of a female-sterile mutant in a population. Consequently the number of perithecia measured reflected the proportion of wild-type female-fertile individuals remaining in the population. Monospore isolation at an intermediate generation confirmed that the evolved strains consisted of mixtures of female-fertile and female-sterile individuals. As several thousands of conidia were transferred between asexual generations, bottlenecks, even existing, were much less stronger as compared to monospore transfer, and favored the invasion, hence the detection, of mutants.

The detection of female-sterile mutants was also likely favored because female-sterile mutants invaded the population, probably due to a selective advantage. In our case, such a fitness advantage was revealed by several experiments where fitness traits were measured between female-fertile and female-sterile strains grown either individually or in competition. Growth and sporulation rates measured individually were not higher for female-sterile than for female-fertile strains, neither *in vitro* nor *in planta*. But for three mutants over the four we tested, grown either separately or in competition in the exact selective conditions of the

evolutionary experiment initial protocol, we showed that conidia of female-sterile were transferred significantly better than conidia of female-fertile strains. This is likely linked to an alteration in the conidia detachment process. For the remaining mutant an alternative hypothesis may apply. Elena & Lenski (2003) reported that in several experiments fitness gains are initially rapid and tend to decelerate over time. This could explain the dynamics of the loss observed in our study: the decrease in the number of perithecia, reflecting the invasion of female-sterile mutants, was initially rapid and slowed down until complete loss of female fertility.

Interestingly, we observed a modification in asexual reproduction via modifications in asexual spores production *in vitro*. One would have expected a trade-off between sexual and asexual reproduction, namely, an increase in asexual spore production when female fertility decreases. However, in our experiment we observed the opposite feature: asexual sporulation decreased in experimental strains that had lost female fertility, at least *in vitro*, and also indirectly *in planta* through a reduction of the number of lesions. This can be explained either by independent mutations or by mutations on a single locus with positive pleiotropic effects on sexual and asexual reproduction. The existence of positive correlation between sexual and asexual reproduction abilities has already been observed in different pathosystems. For example, in an experiment on *M. oryzae* finger millet strain, Tharreau *et al.* (1997) found contrasting results, since female sterility was associated with higher asexual sporulation *in vitro* but lower sporulation *in planta*. Ali *et al.* (2010) found a positive correlation between sexual spores production and asexual spores production. Zeyl *et al.* (2005) found a positive correlation between sexual fitness and asexual fitness in wild-type strains in *Saccharomyces cerevisiae* when sexual selection was applied. A decrease in asexual reproduction ability associated with a reduction or even a loss of sexual reproduction ability *in vitro* was also observed in *Cryptococcus neoformans* (Xu 2004a). Hill and Otto (2007) showed positive pleiotropic effects between sexual sporulation and asexual sporulation in experimental evolution of *S. cerevisiae*. Mycelial growth rate and sporulation rate are often used as fitness values in fungi (Pringle & Taylor 2002). However, we found in the progenies of two crosses, that the decrease in asexual sporulation was independent from female sterility. So, in the particular case of this study we have no evidence of a pleiotropic effect of mutations to sterility on asexual sporulation.

Another interesting result was the decrease in male fertility, that is, the ability of a strain to induce the production of perithecia by another strain. About half of the strains had completely lost this ability at the end of the experiment. The correlation between decrease in male fertility and loss of female fertility observed for some of the evolved strains cases may reflect pleiotropic effects of mutations.

At some point of the experiment, we also observed evolved strains that were able to produce perithecia in the absence of opposite mating type. This trait did not persist during the experiment. Self-formation of sterile perithecia was already observed in one *M. oryzae* isolate from finger millet (Tharreau *et al.* 1997) and the gene responsible for female fertility (already mentioned above) had negative epistatic effects on the gene responsible for self-formation of sterile perithecia. The occurrence of such a phenotype may also be due to mutants that went rapidly extinct because of lower fitness.

Considering that female fertility and hence sexual reproduction can be easily lost, an important question is why some *M. oryzae* strains have remained female-fertile in Asia, in other words which advantage sexual reproduction gives to these strains in this environment. In the laboratory, ascospores were shown to have a short life expectancy and are perithecia unlikely to represent a resting stage *in natura*. As Asia is the centre of domestication of rice (Fuller *et al*. 2009), it is likely that the diversity of varieties is higher in this area than in the rest of the world. In such a heterogenous environment, strains that are able to reproduce sexually may be selected for.

Conclusion

Through experimental evolution, we showed that female fertility could be rapidly lost in *M.* oryzae and that it involved genetic mechanisms rather than epigenetic mechanisms. Experimental evolution of microorganisms is a suitable method to understand different evolutionary mechanisms (Colegrave & Collins 2008), in particular those that could be involved in the loss of sex in several species (Bell 2008). Experimental evolution *in vitro* does not reflect exactly what happens *in natura*, but can be viewed as an approximation of a situation in a favorable constant environment (Souza *et al.* 2002). Here we showed that

female fertility was lost easily and rapidly after few cycles of strictly asexual reproduction. This study brings new insights into the processes underlying the evolution of reproductive mode in *M. oryzae in natura*. Yet, recent studies have shown that, although *M. oryzae* populations are strictly asexual and that female-fertile strains are absent in most of the rice growing areas (Zeigler 1998), this species is still reproducing sexually in its centre of origin in Asia, at least in limited areas (D. Saleh, E. Fournier and D. Tharreau, personal communication). We previously suggested that the sexual reproduction ability was lost during the spread of the pathogen from its centre of origin to the rest of the world, accompanying the host-tracking process during rice domestication and dissemination. This may be due to genetic drift after a bottleneck during the introduction of the fungus in a new area. If one single mating type has been introduced in a new habitat, sexual reproduction was not possible and the fungus reproduced only asexually. Under these conditions, the results of the present study suggest that female fertility could have been lost very rapidly, leading to a complete and definitive loss of sexual reproduction ability.

To disentangle the genetic bases of the phenotypic modifications observed in our experiment, genetic mutations distinguishing the evolved and ancestral genotypes must be identified. The combination of genetic studies and high-throughput sequencing methods may be used to identify the gene(s) responsible for female fertility/female sterility faster. Studying the evolution of polymorphisms of this or these gene(s) between natural female-fertile, sexually reproducing strains from Asia, and natural female-sterile, clonally reproducing strains from other parts of the world, could then help better understanding how reproductive modes have evolved in *M. oryzae*.

Author's contributions

DS, DT and EF designed the study. DS realized the experiment, analyzed the data, interpreted the results and drafted the manuscript. JM and HA contributed to the laboratory work. DT contributed to the laboratory work, the data analyses, the interpretation of the results, and to manuscript writing. EF contributed to data analyses, interpretation of results, and to manuscript writing. All authors read and approved the final manuscript.

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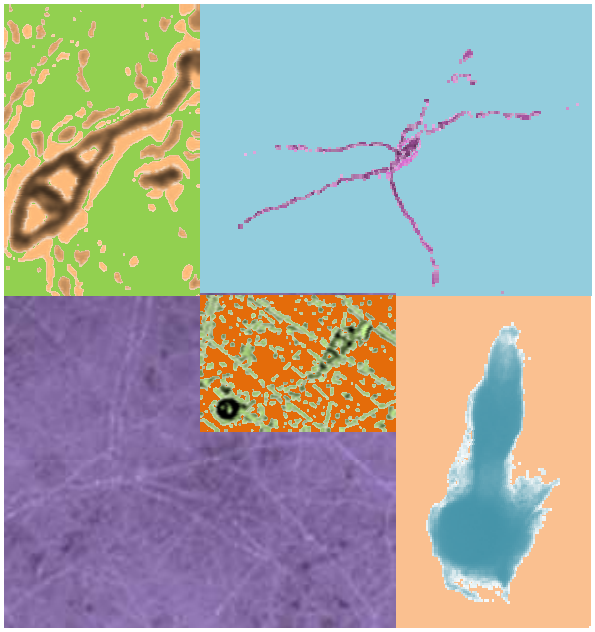
IV. Conclusions sur la partie 2.

Nous avons mis en évidence sans ambiguïté une reproduction sexuée dans une population chinoise. Les valeurs d'indices pour estimer la recombinaison et la distribution des types sexuels et des souches femelle-fertiles suggèrent de la reproduction sexuée dans d'autres régions d'Asie.

Nous n'avons pas pu mettre en évidence de preuves directes. Si la reproduction sexuée a lieu dans la nature, des structures sexuelles doivent être présentes (Milgroom 1996). La preuve ultime de reproduction sexuée, au-delà de l'estimation de la recombinaison, est l'observation directe *in natura*. Chez les champignons ascomycetes, il s'agit de l'observation des périthèces. Or, chez les champignons phytopathogènes, la reproduction sexuée peut avoir lieu sur une plante hôte alternative ou en dehors de sa plante hôte lors d'une phase saprophytique. On parle alors de reproduction sexuelle cryptique. Dans ce cas, l'observation de périthèces *in natura* est plus délicate. Une alternative est d'essayer d'induire la production de périthèces au laboratoire. Celle-ci a été observée chez *M. oryzae in vitro* (Silué & Nottéghem 1990) et *in planta* (Hayashi *et al.* 1997). Si l'on observe des souches capables de se reproduire sexuellement au laboratoire et d'autres qui en sont incapables, il est très fortement probable que les premières soient aussi capables de reproduction sexuée dans la nature si elles rencontrent les conditions favorables. La survie des spores asexuées de *M. oryzae* pendant l'hiver étant très faible (Harmon & Latin 2005), les structures sexuées (périthèces) pourraient constituer une forme de survie.

Nous avons montré que la capacité de reproduction sexuée a très probablement été perdue pendant la dispersion du champignon depuis l'Asie vers le reste du monde par des mécanismes génétiques.

CONCLUSION GENERALE ET PERSPECTIVES



Cette thèse avait pour objectif de mieux comprendre et localiser les évènements importants dans l'évolution de *M. oryzae,* champignon pathogène du riz domestiqué. Nous avons donc déterminé l'origine du champignon en prenant soin de faire la distinction entre le centre d'origine, de diversité et de dispersion. Nous avons ensuite caractérisé le mode de reproduction dans différentes régions du monde.

Centre d'origine, centre de diversité génétique et centre de dispersion de *M. oryzae.*

Par différentes approches de génétique des populations, nous avons confirmé l'hypothèse de l'origine de *M. oryzae* sur le riz cultivé en Asie du sud-est. Ainsi, nous avons montré que son centre d'origine correspondait à la zone de domestication du riz. *M. oryzae* peut être apparu sur le riz cultivé par *host-tracking*, c'est-à-dire par la domestication de l'agent pathogène avec son hôte, ou par saut d'hôte depuis une autre plante. Pour tester ces hypothèses, il sera nécessaire d'étendre l'échantillonnage dans le centre d'origine présenté dans cette thèse à des souches de *M. oryzae* pathogènes d'autres plantes hôtes, en particulier du riz sauvage, *O. rufipogon*, mais aussi de sétaires puisqu'un saut d'hôte a été suggéré depuis cette plante. Il peut y avoir eu saut d'hôte depuis sétaire sauvage sur riz sauvage et *host-tracking* pendant la domestication du riz. Il sera donc nécessaire de dater les divergences entre les souches pathogènes du riz et les souches pathogènes de sétaires afin de déterminer si l'émergence de *M. oryzae* sur le riz est antérieure ou postérieure à la domestication.

La région asiatique qui a été étudiée dans cette thèse s'étendait du Népal à l'est de la Chine en longitude et de la région du Hunan en Chine à l'Indonésie en latitude. L'analyse de marqueurs génétiques neutres à mis en évidence deux centres de forte diversité génétique dans cette zone. Le premier centre correspond à la région du sud du Yunnan en Chine, du Laos et de la Thaïlande et le deuxième centre est situé au Népal. Ces deux centres correspondent aux deux aires présumées de domestication du riz.

Les analyses du chapitre 1 ont apporté une connaissance précise de la répartition de la diversité génétique de *M. oryzae*. La diversité génétique observée en Asie était représentative de la diversité génétique mondiale puisque les clusters génétiques observés en Europe, en Amérique du nord, en Amérique du sud et à Madagascar pouvaient être rattachés à des clusters asiatiques. Ainsi, nous avons montré que l'Asie était le centre de dispersion de *M. oryzae*. Cette étude a aussi permis d'émettre des hypothèses sur les évènements de dispersion depuis l'Asie vers le reste du monde. Nous avons trouvé une diversité génétique et une structuration géographique faibles en Europe et dans le Bassin Méditerranéen, dénotant une

forte homogénéité entre les différents pays et pouvant s'expliquer par une introduction unique depuis l'Asie dans cette aire. Nos résultats sur les populations de Madagascar et d'Amérique du sud sont aussi compatibles avec une introduction unique. En revanche, nous avons trouvé aux Etats-Unis une structure génétique compatible avec l'hypothèse d'introductions multiples depuis l'Asie mais aussi depuis l'Europe. D'une manière plus générale, notre étude soutient l'hypothèse de migrations à longue distance. A partir de ces observations, il sera nécessaire de tester et dater différents scénarios sur les routes de dispersion du champignon (Figure 8.1). Cela pourra être réalisé par l'utilisation de méthodes d'*Approximate Bayesian Computations* (*ABC*) récemment développées et utilisées pour déterminer les routes de dispersion sur plusieurs organismes, dont les champignons phytopathogènes. L'utilisation de marqueurs à taux de mutation moins élevés que les microsatellites, comme par exemple les SNPs, permettra aussi de construire des phylogénies et des réseaux d'haplotypes afin d'étudier des évènements évolutifs plus anciens. Il sera nécessaire de tester l'hypothèse de dispersion anthropique et en tenant compte d'informations historiques sur la culture du riz. La forte homogénéité dans les zones introduites peut s'expliquer par des évènements stochastiques de goulots d'étranglement lors de l'introduction mais aussi par une sélection par l'hôte. Cette dernière hypothèse pourra être testée par la comparaison de mesures de fitness de différentes souches mondiales sur leur hôte et sur d'autres génotypes d'hôtes.

Enfin, dans cette même étude, la présence des deux types sexuels, de souches femelle-fertiles et la forte diversité génotypique ont suggéré une reproduction sexuée en Asie et à l'inverse une reproduction exclusivement clonale à l'échelle mondiale. Nous avons vérifié cette hypothèse par des analyses d'estimation de la recombinaison dans le deuxième chapitre de la thèse.

Evolution du régime de reproduction

Déterminer le régime de reproduction d'un organisme est nécessaire pour comprendre la structuration génétique dans son aire de distribution. Chez les organismes ayant des régimes de reproduction variables, il est important de différencier les populations se reproduisant de manière asexuée des populations se reproduisant de manière sexuée, voire des populations alternant les deux types de reproduction. En effet, le régime de reproduction joue un rôle important dans la structuration génétique des populations. Les champignons sont des organismes qui ont des systèmes de reproduction complexes puisqu'ils peuvent se reproduire de manière sexuée et asexuée. Ainsi, les études de structuration génétique de champignons

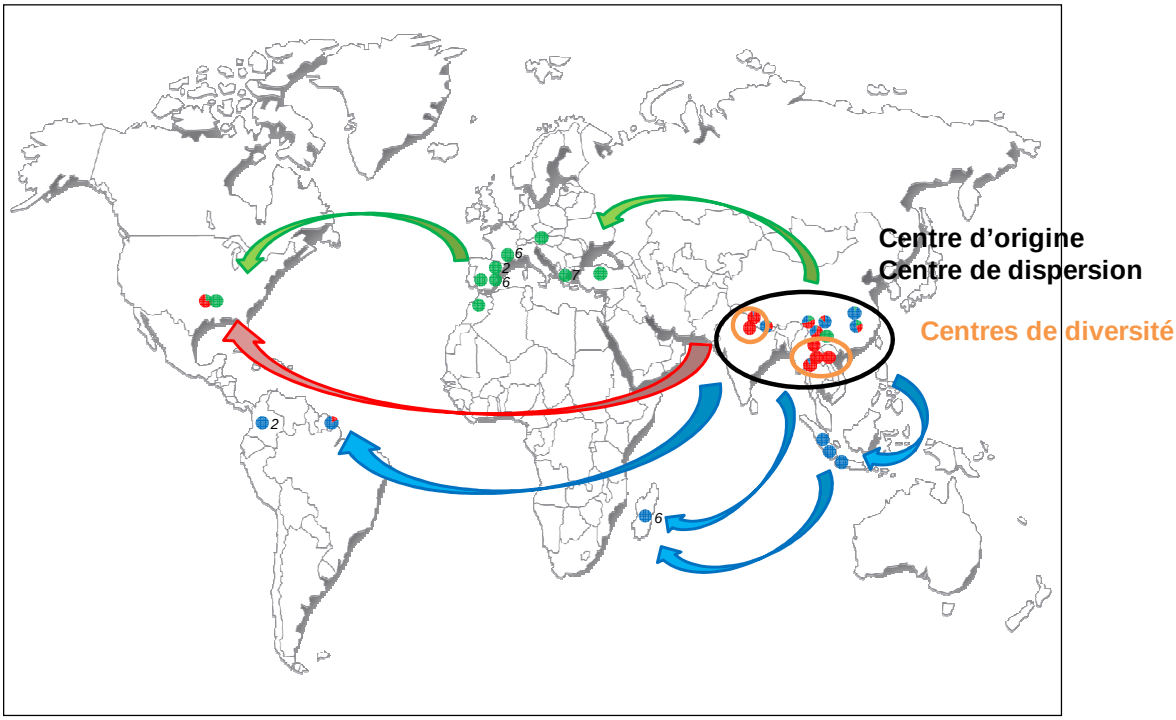


Figure 8.1. Quelques scénarios à tester sur la dispersion de *M. oryzae* depuis son centre d'origine vers le reste du monde.

phytopathogènes intègrent de plus en plus l'estimation de la recombinaison et la distribution des types sexuels. *M. oryzae* est un modèle adapté à ce type d'étude puisque la reproduction sexuée nécessite non seulement des souches de types sexuels opposés mais aussi des souches femelle-fertiles. Dans cette étude il a donc été nécessaire de combiner les approches biologiques et de génétique des populations pour déterminer la capacité de reproduction sexuée et estimer la recombinaison. Ainsi, sur un sous-échantillon représentatif des populations du chapitre 1, nous avons mis en évidence une capacité de reproduction sexuée en Asie. Nous avons montré que les deux types sexuels nécessaires à la reproduction étaient présents dans une même population en Asie. De plus, nous avons mis en évidence l'absence de souche femelle-fertile en dehors de l'Asie alors que certaines populations asiatiques n'étaient composées quasiment que de souches femelle-fertiles. En augmentant le nombre de marqueurs par rapport à l'étude du chapitre 1 nous avons amélioré la probabilité de détecter des évènements de recombinaison. Ainsi, des évènements de recombinaison ont été détectés dans une population du sud du Yunnan en Chine, correspondant au centre de diversité génétique précédemment discuté. En comparaison, la probabilité de recombinaison était faible et la capacité de reproduction était nulle dans des populations d'Europe, d'Amérique et de Madagascar. Ce travail de thèse apporte donc des éléments nouveaux par rapports aux études précédentes, puisque nous nous sommes placés à la véritable échelle à laquelle la reproduction sexuée peut avoir lieu: le champ. Les simulations ont aussi permis d'obtenir une enveloppe de valeurs théoriques sur la proportion de génotypes multilocus dans un échantillon et le déséquilibre de liaison multilocus en l'absence de recombinaison. Cette approche a permis de simuler des conditions proches de celles de l'organisme étudié et de les comparer aux données réelles. Malgré l'absence de preuves directes, le faisceau de preuves indirectes que nous avons fournies dans cette thèse permettent de conclure que la reproduction sexuée de *M. oryzae* a bien lieu au moins dans une population du sud du Yunnan en Chine, et est susceptible d'exister dans différentes zones de l'Asie. Nous avons cherché des preuves directes, c'est-à-dire des périthèces lors d'une mission en Chine, notamment dans cette population. Nous n'avons pas pu approfondir les recherches à ce moment là mais à l'avenir il sera nécessaire de mettre en place un suivi des populations de *M. oryzae* pendant une année pour déterminer quand et où a lieu la reproduction sexuée.

Nos résultats ont aussi confirmé l'hypothèse de perte de la reproduction sexuée depuis le centre d'origine, de diversité et de dispersion, l'Asie, vers le reste du monde. Nous avons

testé la pertinence de cette hypothèse par une approche d'évolution expérimentale *in vitro* de quatre souches femelle-fertiles issues de la population chinoise dans laquelle nous avions détecté la reproduction sexuée. Nous avons observé la perte de fertilité femelle, composante indispensable à la reproduction sexuée chez ces souches, par des mécanismes génétiques. Dans plupart des cas, les spores asexuées étaient transmises plus facilement entre deux générations clonales quand les souches avaient perdu la fertilité femelle. Nous avons donc montré que la reproduction sexuée pouvait avoir été perdue par des mécanismes génétiques lors de la dispersion du champignon. Par la suite, il sera primordial d'identifier les mutations responsables de cette perte de fertilité et de séquencer des souches femelle-fertiles et femelle-stériles d'Asie et du reste du monde pour déterminer si ce sont bien les mêmes mécanismes qui ont été impliqués dans la nature. Plusieurs hypothèses restent à tester pour déterminer les évènements qui ont conduit à une perte de la capacité de reproduction sexuée en dehors de l'Asie. Dans certaines régions du monde, la dérive génétique peut avoir éliminé les individus femelle-fertiles ou alors un type sexuel. Dans ce cas, la perte de capacité de reproduction sexuée serait stochastique. C'est une explication possible pour la région comprenant l'Europe et le Bassin Méditerranéen puisque nous avons montré une origine unique de toutes les souches provenant de cette région et que nous n'y avons détecté qu'un seul type sexuel, Mat1. Une autre hypothèse possible pour expliquer la perte de reproduction sexuée est l'homogénéité de l'hôte dans les zones introduites qui a favorisé la sélection pour la clonalité.

***Magnaporthe oryzae* : modèle de co-évolution hôte pathogène et modèle en biologie évolutive.**

Il est de plus en plus reconnu que l'étude des interactions entre hôte et parasite dans un milieu domestiqué nécessite une stratégie intégrative, c'est-à-dire une combinaison de plusieurs approches allant de l'étude fonctionnelle des gènes impliqués dans l'interaction à l'étude des processus évolutifs. Cette thèse qui s'inscrit dans un registre fondamental, n'a pas prétention à proposer des solutions dans la lutte contre la pyriculariose, mais elle peut apporter des éléments à prendre en compte.

Ainsi, la domestication du riz et l'intensification de l'agriculture et des échanges commerciaux ont eu des conséquences évolutives importantes sur *M. oryzae* qu'il est primordial de comprendre pour améliorer la lutte et anticiper de futurs changements évolutifs. Considérant le spectre de virulence réduit de chaque lignée de *M.* oryzae, l'exclusion de lignées par la culture de variétés de riz résistantes a été proposée comme stratégie de lutte.

Cette méthode n’est efficace que si la reproduction du champignon est clonale puisque la recombinaison permet de combiner des virulences entre différentes lignées. Il est donc important, dans les populations où la reproduction sexuée existe (nous l’avons montrée en Chine), de déterminer si les lignées de *M. oryzae* sont plus diverses et susceptibles de favoriser l’apparition de souches multi-virulentes dans un champ.

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Annexes

Annexe 1

Population structure of the rice blast fungus in Europe at different geographic scales.

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Summary

- *Magnaporthe oryzae* is the causing agent of the main fungal disease of rice, blast and is distributed worldwide.
- In this work, 987 blast isolates were collected in seven rice producing European countries. Their genetic diversity was investigated using 11microsatellite markers.
- The European blast population is divided in three major genetic groups, not previously identified and that cannot be explained by geography only. The most common multilocus genotypes (MLGs) are shared between European countries. However, we show that some MLGs are specific to different countries, and even to different rice growing areas within countries. The genotypic diversity measured in farmer's fields reveals that, although one or two genotypes are dominant, several genotypes frequently coexist. The hypothesis of weedy rice as a source of primary inoculum cannot be excluded. *M. oryzae* genetic evolution was investigated over the last 25 years in two countries. In France, significant population changes were observed over time, contrarily to Italy.
- Our data pinpoint that there are important gene flows between European rice growing areas, probably through transportation of infected seeds. We also show evidence of differentiation at a very small geographic scale, suggesting local adaptation.

Key words: *Magnaporthe oryzae*, rice, genetic diversity, Europe, migration, selection

Introduction

Magnaporthe oryzae is a fungal pathogen causing blast disease on rice and other Poaceae. The disease is distributed worldwide including in temperate rice growing regions like Europe or Japan. Selection and cultivation of resistant rice varieties is one of the most efficient and cost-effective strategies to control this disease. But most varieties resistant to blast are attacked by new virulent races two to six years after their release (Bonman *et al.*, 1992). Part of this failure to breed for durable resistance is due to our limited understanding of the dynamics and evolution of the rice blast fungus populations.

Numerous population studies of the rice blast fungus have been conducted (Levy *et al.*, 1991; Han *et al.*, 1993; Levy *et al.*, 1993; Xia *et al.*, 1993; Correa-Victoria *et al.*, 1994; Chen *et al.*, 1995; Roumen *et al.*, 1997; Don *et al.*, 1999a; Don *et al.*, 1999b; Correll *et al.*, 2000; Xia *et al.*, 2000 ; Park *et al.*, 2003 ; Javan-Nikkah *et al.*, 2004 ; Park *et al.*, 2008 ; Laura-Alvarez *et al.*, 2010). They have allowed characterizing or confirming biological features of this species. Population studies have shown that this pathogen reproduces clonally in most rice growing areas with exceptions in the putative Centre of origin (Zeigler, 1998; Kumar *et al.*, 1999; Tharreau *et al.*, 2009; D. Saleh, E. Fournier, D. Tharreau, unpublished). Worldwide analysis also suggests intercontinental migration (Tharreau *et al.*, 2009). At the European scale, preliminary population studies revealed that there are few clonal lineages (6-7), half of them being specific of one country, the other half being present in most countries (Jorge 1996; Roumen *et al.*, 1997; Piotti *et al.*, 2005; Lara-Alvarez *et al.*, 2010). These results confirm migration between relatively distant rice growing areas (several hundred km). Since *M. oryzae* spores disseminate only on short distances (Nottéghem, 1977), long distance migration is suspected to result from transportation of infected seeds (Tharreau *et al.*, 2009).

But many questions were not really addressed and important biological characteristics remain poorly analyzed. Migration, survival, and even diversity of the pathogen were not well

evaluated. In previous studies, the sample size and sampling scheme did not really allow to characterize the European population structure and to evaluate the importance of migration. More generally, the population structure and diversity at the field scale is poorly documented, especially in farmer's fields. Published population studies of the blast fungus are often based on collection of samples in experimental fields where different varieties are grown. Rice varieties carry different resistance genes that exclude specifically some blast strains. Thus, the population structure observed is linked to the rice varieties sampled. In addition, very few appropriate samplings with a sufficient number of strains collected from the same rice variety were realized to measure the diversity at the field scale. Among these few studies, a survey in Korea showed a surprisingly high genotypic diversity in rice fields since almost all isolates had a unique multilocus genotype (MLG; Park *et al.*, 2008). On the contrary, in two farmer fields sampled intensively in Arkansas (USA), Xia *et al.* (2000) showed that several MLGs were present in each field but that either one or two of them dominated. Population evolution over time is also poorly documented. Lineages did not change for 20 years while seven dominant varieties occupied 70 % of the rice fields during the same period in Korea (Park *et al.*, 2003). In Arkansas, over 17 years, the general trend of evolution was from four to two clonal lineages (*sensu* Levy *et al.*, 1991), one being dominant (95%; Correll *et al.*, 2009). But, important inter-annual variations were also observed elsewhere. In Japan, original lineages disappeared after varietal changes (Don *et al.*, 1999b). In Southern Spain, drastic inter-annual changes of clonal lineages were also observed (Lara-Alvarez *et al.*, 2010) but could not be explained.

In temperate areas, there is no rice grown during more than six months. So, unlike in some tropical rice growing agrosystems, rice blast epidemics are stopped during winter. Infected seeds were shown to be a potential source of primary inoculum (Long *et al.*, 2001). Overwintering on rice straw or in the soil is difficult to test but seems unlikely because *M.*

oryzae does not have resting structures able to survive cold and competition with microorganisms. Rice blast, as a species, is pathogenic to different plant hosts. But, strains pathogenic to rice are specialized and there is no demonstration that populations from rice cause epidemics on weeds *in natura*. Thus, the hypothesis of overwintering on an alternative and different host species is also unlikely in temperate areas. However, weedy rice (also called red rice) occurs very frequently in cultivated rice in Europe. Because weedy rice and cultivated rice belong to the same species (*Oryza sativa*), *M. oryzae* rice strains are pathogenic to weedy rice. Thus, this species could serve as a primary inoculum source in temperate areas.

We characterize the diversity of a collection of more than 980 strains collected in 7 European countries with microsatellite markers. This collection gathered strains isolated over the last 20 years, including recent samples designed to investigate specific questions. We then exploited these data to try to address the following questions:

- What is the population structure of the rice blast fungus in Europe? Are migrations between rice growing areas so frequent that it can be considered as one population?
- How these populations changed over the last 20 years?
- What is the diversity at the farmer's field scale?
- What is the primary source of inoculum? We tested in particular the hypothesis that weedy rice could be the primary source of inoculum.

Material and Methods

Sample collection and blast isolates

Isolates were collected from blast-infected rice samples (leaf, neck, rachis and seed) during different years (from 1986 to 2009) from various rice varieties grown in seven different European countries: France, Greece, Hungary, Italy, Portugal, Spain, and Turkey. In addition, a specific sampling was realized at the field level: 10-30 strains from single farmer's fields were isolated in France, Greece, Hungary, Spain and Turkey. In France, infected weedy rice plants were also sampled in parallel to the cultivated ones in several fields. Strain isolation was done as previously described (Lara-Alvarez *et al.*, 2010). Briefly, samples showing blast symptoms were placed onto watered sterilized filter paper in 9-cm Petri dishes and then incubated at 27°C in a moist chamber with alternating 12-h periods of fluorescent light for 1 to 2 days. A single conidium was isolated from each sporulating sample and cultured at 27°C on water agar medium (40 g Bacto-Agar in 1 l water) overnight. Germinated conidia were transferred to 9-cm Petri dishes containing rice-polish agar medium as previously described (Silué *et al.*, 1992). For strain storage, cultures were grown on rice-polish agar medium, but covered with sterile filter paper. When colonized by the fungus, the paper was removed, dried and vacuum-sealed in plastic bags prior to storing at -20°C as described in Valent *et al.* (1986).

Eleven *M. oryzae* strains representative of the five European lineages described by Roumen *et al.* (1997) were used as reference isolates (isolates FR13, IT10 for lineage E1, IT02, PR03, PR72 for E2, HN01 for E3, SP05, SP06 for E4 and FR27, FR28, IT03 for E5).

DNA extraction and fingerprint analysis

Total genomic DNA was extracted from 987 European isolates: 476 French (FR), 208 Italian (IT), 183 Spanish (SP), 68 Greek (GR), 24 Hungarian (HN), 20 Turkish (TR), and 8 Portuguese (PR). DNA extraction was carried out as already described (Lara-Alvarez *et al.*,

2010). Among the 18 microsatellite markers previously developed by our group (Adreit *et al.*, 2007), we chose, on the basis of allele number revealed, 11 primer pairs (Table 9.1) with a new design for 2 primers (77 to 77B and 84 to 84B; 77B: AGGCTCTCTGCCTACGAAGT, 84B: GCAAAGTTGTTTGAGCAAGG) in order to get a different size range. Three pools of three primer pairs and one pool of two were used for multiplex PCR which was performed according to Adreit *et al.* (2007). PCR products were then separated on a 16-capillary sequencing machine (ABI Prism 3130XL, Applied Biosystems, Foster City, USA), after mixing 1 µL of diluted (1/100) amplified products with 12 µL of Formamide GeneScan-500LIZ size marker (Applied Biosystems, Foster City, USA).

Genotyping data analysis

Alleles were identified using the GeneMapper® software (Applied Biosystems, Foster City, USA). The number of different multilocus genotypes (MLGs) was calculated using an Excel macro developed by Jean Peccoud. Population structure for the 125 distinct MLGs identified and the 21 genotypes that could not be attributed unambiguously to a defined MLG due to missing data was first inferred using the model based clustering program STRUCTURE v2.2 (Pritchard *et al.*, 2000; http://pritch.bsd.uchicago.edu/structure.html). The model we used in this study allows for admixture and correlated allele frequencies. To find the best number of clusters (K), we tested three independent runs for a continuous series of K values (1–10) with a burn-in of 10,000 iterations and a run length of 100,000 iterations. The mean values of three log-likelihoods for each K were then plotted to find the minimum K when likelihood value stabilizes. The DARWIN phylogenetic analysis software (http://darwin.cirad.fr/darwin; Perrier *et al.*, 2003; Perrier and Jacquemoud-Collet, 2006)

Marker name	Repeat motif	Primers	Multiplex pool	Allele size range (bp)
Pyrms37-38	$CA/GT_{6+}CT/GA_{12}$	F: (NED)-ACCCTACCCCCACTCATTTC	4	188-278
		R: AGGATCAGCCAATGCCAAGT		
Pyrms47-48	TA_{15}	F: (FAM)-TCACATTTGCTTGCTGGAGT	1	156-195
		R: AGACAGGGTTGACGGCTAAA		
Pyrms63-64	CT/GA_{15}	F: (NED)-TTGGGATCTTCGGTAAGACG	3	149-177
		R: GCCGACAAGACACTGAATGA		
Pyrms77B-78	CA/GT_{24+}	F: (PET)-AGGCTCTCTGCCTACGAAGT	2	220
		R: GCTTTCGGCAAGCCTAATC		
Pyrms83-84B	TCA/AGT_{13}	F: (PET)-GTCTGCCTCGACTCCTTCAC	3	112
		R: GCAAAGTTGTTTGAGCAAGG		
Pyrms233-234	CAG/GTC_{10}	F: (FAM)-TGAGATGGACCGCATGATTA	4	239-283
		R: TTGATGGCAGAGACATGAGC		
Pyrms319-320	CAA/GTT_{6}	F: (NED)-TAAGACCACTGGCGGAATCT	4	283-292
		R:GGCTTTGTCTGGTTGTACGG		
Pyrms409-410	TA/AT_{23}	F: (FAM)-TCCCAGTACTTGCCCATCTC	2	311
		R: CTCCGATTCATGGCACACAC		
Pyrms427-428	AT/TA_{16}	F: (VIC)-CTGTCACCACAACCAAGACG	1	196-242
		R: TTGCCCTGATTTGTCAGTCA		
Pyrms607-608	GCA/CGT_{13}	F: (VIC)-CCCAAGCTCCATAATACGCTAC	3	271-302
		R: TCCGAGACTCTTTGGATAGCAC		
Pyrms657-658	CA/GT_{12}	F: (VIC)-ATCAGTCGAACCCACAAAGC	2	161-171
		R: ATGTGTGGACGAACCAGTCC		

Table 9.1. Primer description and allele size range for the 11 microsatellite markers used for study of European genetic diversity of *Magnaporthe oryzae.*

allowed us to estimate the genetic distance between MLGs from allelic matrices using simple matching coefficient. All MLGs were analyzed through the Factorial Analysis method implemented in DARWIN. Finally, a Minimum Spanning Tree of MLG was constructed with ARLEQUIN 3.5.1.2 (Excoffier &, Lischer, 2010) and visualized with HAPSTAR 0.6 (Teacher & Griffiths, 2011).

Results

M. oryzae genetic diversity and structure at the European scale

The genotypic diversity of 987 *M. oryzae* strains, sampled in seven European rice-producing countries, with 11 independent microsatellite markers allowed their assignment to 125 multi-locus genotypes (MLGs). In the sampled population, nine of them were observed at least 40 times, representing 58% of the total number of strains (Figure 9.1). The most frequent genotype (MLG14) was detected 107 times (11%). Eleven of the MLGs were repeated between 10 and 19 times, 45 between 2 and 9 times and 60 only once (Figure 9.1). Due to missing data, 21 genotypes could not be attributed unambiguously to a defined MLG and were classified as non-determined (nd). The six most frequently occurring genotypes (MLG14, 1, 39, 3, 21, and 7) were present in the majority of European rice-producing countries, i.e. France, Italy, Greece, Spain and Portugal. At the same time, some genotypes were specifically found in only one of those countries, such as MLG9 and 107 observed 43 and 41 times in France and in Spain, respectively. In Turkey, two major genotypes (MLG103 and 115) were observed 14 and 3 times respectively. MLG103 was detected in Portugal for one isolate as well. Three other minor genotypes were also observed once in that country (MLG88, 113, and 114). In Hungary, *M. oryzae* strains resulted in two specific frequent

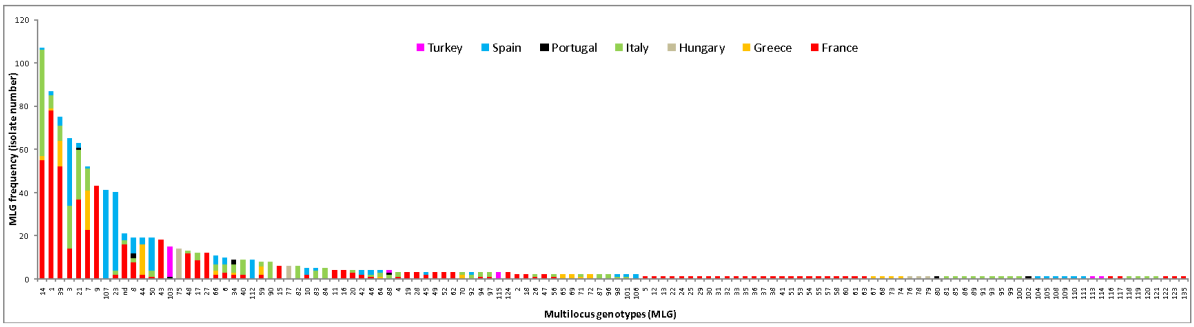
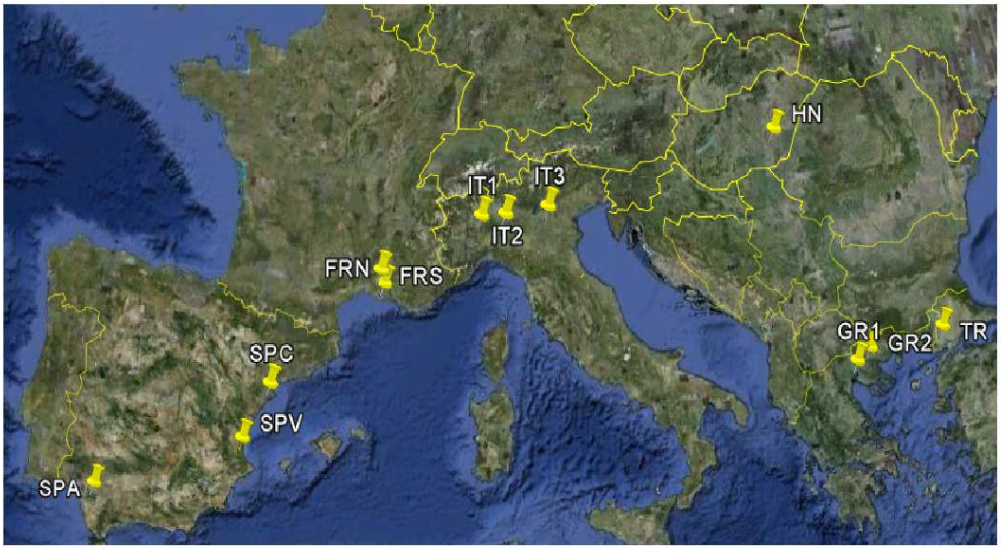


Figure 9.1. Distribution of multilocus genotypes (MLGs) representative of *Magnaporthe oryzae* genetic diversity in seven European countries: France, Greece, Hungary, Italy, Portugal, Spain and Turkey.



Supplementary Figure S9.1. Sampling locations of *Magnaporthe oryzae* populations in Europe. The following populations were sampled for this study: Spain – Andalucía (SPA), Valencia (SPV), Ebre delta (SPC); France – North Camargue (FRN), South Camargue (FRS); Italy – Piemonte-Lombardy (IT1 and IT2), Veneto (IT3), Hungary (HN); Greece – Thessalonik area (GR1 and GR2); Turkey (TR).

genotypes: MLG75 and 77 repeated 14 and 6 times. In addition, four other minor MLGs, represented by a single strain, were also detected (MLG76, 78, 79, and 80). So, unlike in other countries, all the MLGs found in Hungary were specific of this country.

From this large set of data we obtained emerge clear evidence that the most common MLGs are shared between all European countries, as suggested by previous preliminary results (in Jorge, 1996 and Roumen *et al.*, 1997). However, we show that there are some country-specific MLGs, suggesting some differentiation between countries in Europe.

Genetic diversity within countries

To investigate the existence of local differentiation, we examined the distribution of MLGs at the regional level within countries. In France, two regions of samplings were defined (Northern and Southern Camargue, 30km away from each other; Supporting Information Figure S9.1). Our analysis revealed that the distribution of MLGs was different between the two sampling areas (Chi^2 test, $P<0.0001$). Some genotypes were significantly over-represented or even specific to Northern Camargue (MLG14, 7, 17 and 48) or to Southern Camargue (MLG1, 39, 21 and 9; Figure 9.2a). In Spain, Greece and Italy, blast infected samples were also collected in different areas (Supporting Information Figure S9.1), at least 50 km away from each other. In Spain, samplings were carried out in three different regions: Andalucía in the South (Guadalquivir delta), Cataluña (Ebre delta) in the North, and Valencia (Albufera lagoon area) in between. Figure 9.2b shows that the three most frequently observed genotypes (MLG107, 23, and 3) were found in the three Spanish sampling areas (MLG107) or in Valencia and Cataluña (MLG23 and 3). On the other hand, some MLGs were specific to Andalucía such as MLG66, 44, and 106. In Greece, blast infected samples were collected in two regions in the North: Thessaloniki area and Northern-East Thessaloniki

(Supporting Information Figure S9.1). All genotypes were either specific (MLG44) or predominantly found in one region (MLG7 and 59 or MLG39; Figure 9.2c). In Italy, the samplings were carried out in three different areas: two sites in Lombardia-Piemonte at a distance of 70 km and the third in Venetia (Supporting Information Figure S9.1). The most frequent genotype (MLG14) was detected in the all three areas and no region-specific MLGs were significantly detected (Figure 9.2d).

To further characterize the diversity at the sub-regional scale, fungal strains from four fields sampled in France in 2006 and in 2009 were compared. The MLG composition was significantly different from one plot to another (Figure 9.3). The total number of MLGs per field ranged from four to eight. One and two MLGs represented more than 50% of the total number of strains in three (Vigne - Euro, Chartrousse - Ambra and Juge) and four (Gimeau, Manusclat, Chartrousse - X, Eyselle) fields, respectively. Two MLGs were found in three plots in 2006 (MLG14 and 17) and 2009 (MLG1 and 43), but they were different between years and their frequencies differed significantly between fields. The second dominant MLG was also different in these plots. The comparison of strains sampled on the same site (Chartrousse) but from two different varieties (Ambra and an unidentified but different variety) also showed significant differences for MLG distribution. On Ambra, five MLGs were detected, of which MLG1 represented 80% of the strains. On the other variety, grown in an adjacent field, six MLGs were observed. MLG1 was also frequent but was significantly less represented (40%) and codominated with MLG39 (Figure 9.3). We therefore show a marked geographic structure, even at a small scale (20,000 ha of rice-growing area distributed over 150,000 ha in total for Camargue) and a strong impact of the variety of origin on the population structure.

MLGs specific to different rice growing areas were observed within countries. This result suggests local differentiation. Such differentiation may be due to difference in inoculum

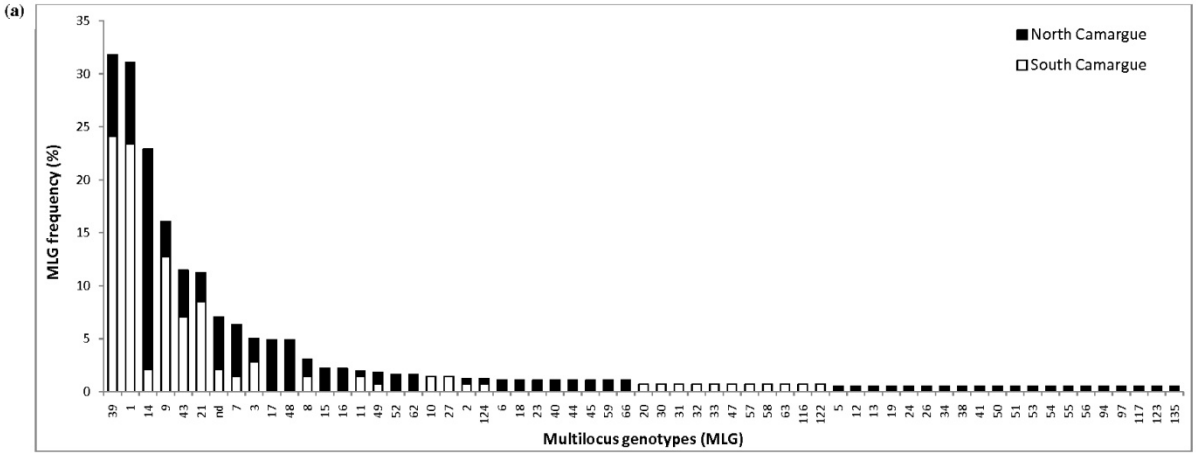


Figure 9.2. Distribution of multilocus genotypes (MLGs) representative of *Magnaporthe oryzae* genetic diversity within France, Spain, Greece and Italy. (A) French rice-growing area, Camargue (North and South); **(B)** Spanish rice-growing areas, Ebre delta (North), Valencia (Center) and Andalucía (South); (C) Greek rice-growing areas near Thessaloniki (GR1) and Northern-East Thessaloniki (GR2); (D) Italian rice-growing areas, Piemonte-Lombardy (IT1, IT2) and Veneto (IT3).

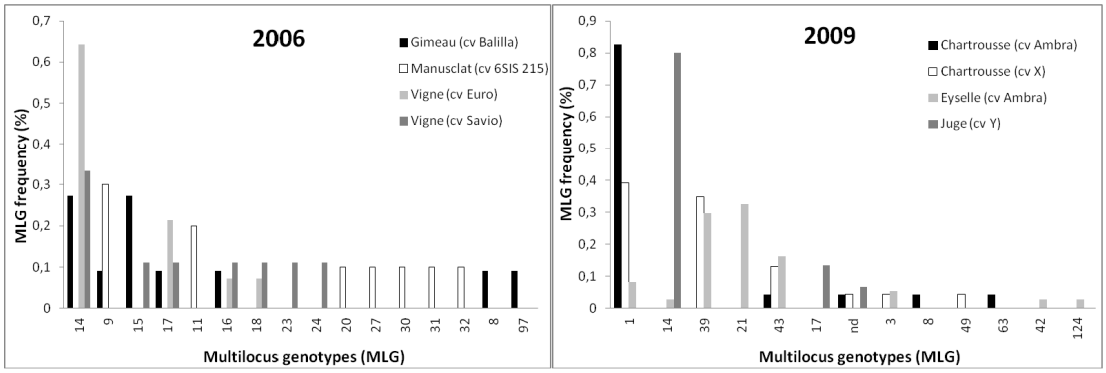


Figure 9.3. Genotypic diversity in six farmer's fields in France. Each field is designed by the name of the pace and the name of the variety into brackets (X or Y = undetermined variety name).

sources (seed lots), in rice varieties with different levels of resistance cultivated in those areas or in agro-environmental conditions or combinations of these factors with limited gene-flow between fields.

Genetic diversity at the field level

The genetic diversity was assessed at the field level. For this study, only samples consisting of more than 10 strains collected in farmer's fields and from plants of a single variety per field were considered. Seven samples from France, five from Greece, five from Spain and one from Turkey were analyzed. The genotypic diversity ranged from 0.31 to 0.82 with an average value of 0.62 (Table 9.2). The number of MLG per field varied between three and eight with an average of 4.9 (for an average sample size of 15.9; Table 9.2). In the majority of these populations, one or two MLGs represented more than 50 % of the strains (nine and eight populations respectively over 18 populations). The MLGs encountered in one field were genetically more related than the MLGs encountered over Europe, as shown by the distribution of the average distances between MLGs (Supporting Information Figure S9.2). This suggests that in one field, the different genotypes detected mostly derive from one to the other by mutations.

Weedy rice vs. cultivated rice

To test whether weedy rice is a potential source of inoculum to cultivated rice, we compared population structures on these two hosts in France. We used samples collected in recent years (2006 to 2009) on both hosts in the same field or in contiguous fields. If weedy rice is the source of inoculum, it is expected that the population structures will be similar.

	FR1	FR2	FR3	FR4	FR5	FR6	FR7	GR1	GR2	GR3	GR6	GR7	SP1	SP2	SP3	SP4	SP5	TR1	Average
Sample size	10	11	13	15	23	23	37	10	10	10	10	10	12	13	13	18	29	19	15.9
No of MLG	7	7	3	3	5	6	8	6	7	4	4	3	7	3	6	3	3	4	4.9
Genotypic diversity	0.82	0.81	0.46	0.34	0.31	0.71	0.77	0.80	0.82	0.70	0.58	0.58	0.81	0.38	0.78	0.54	0.47	0.43	0.62
Frequency of most frequent MLG	0.30	0.27	0.69	0.80	0.83	0.39	0.32	0.30	0.30	0.40	0.60	0.50	0.33	0.77	0.31	0.61	0.66	0.74	0.51

Table 9.2. Genetic diversity of *Magnaporthe oryzae* within 18 fields in Europe.

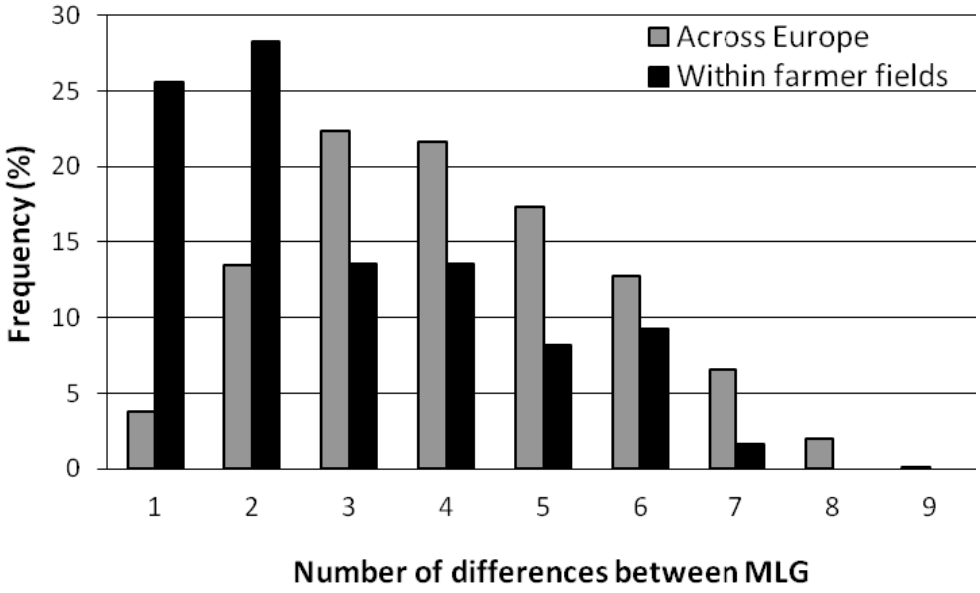


Figure S9.2. Genetic relatedness between multilocus genotypes (MLGs) across Europe and within farmer's fields (average of 18 fields sampled in France, Greece, Spain and Turkey).

Globally, the distribution into the different MLGs was not significantly different (Chi² test, P = 0.95; Figure 9.4). Fifteen MLGs were common to both hosts. Twenty-four and 12 MLGs were sampled only on cultivated rice and only on weedy rice respectively. But most of these specific MLGs represented less than 2.6% of the total number of strains, and were probably not detected for one or the other host because of this low level of occurrence. These data suggest that populations on both hosts are similar and that weedy rice may represent a source of primary inoculum. However, five MLG frequencies were significantly different between the two hosts (Chi² test, P<0.01). Indeed MLG7, 21 and 27 were significantly over-represented on weedy rice and MLG39 and 43 were over-represented on and unique to cultivated rice, respectively (Figure 9.4). This may suggest some specificity. To test this hypothesis, and because the overall genetic structure may not reflect local structure, we compared MLG frequencies for strains isolated from the same field. Four fields were analyzed. In three fields (1, 3, 4; Figure 9.5), one or two MLGs were significantly over-represented on one host or the other. These included MLG7, 39 and 43 previously detected (see above). However, in each field, the overall distribution of MLGs was not significantly different between samples collected on weedy and on cultivated rice. In three fields (1, 2, 4; Figure 9.5) the dominant MLG was the same on both hosts. Based on these results, it seems that weedy rice and cultivated rice host the same strains. So, the hypothesis of weedy rice being a source of primary inoculum for cultivated rice cannot be excluded.

Evolution of M. oryzae polymorphism over the last 25 years

In France and in Italy, blast infected samples have been regularly collected since 1986. We therefore decided to look at MLG evolution through those 25 years in these 2 countries. Although sample size is quite unbalanced, we attempted to study population evolution over

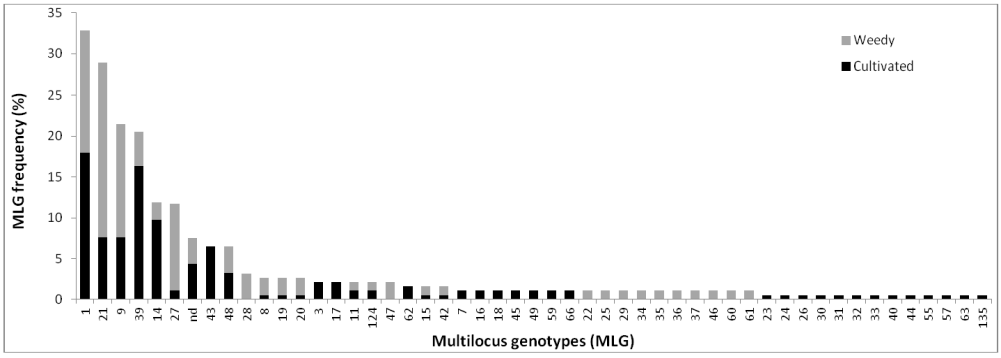


Figure 9.4. Genotypic diversity observed in France on cultivated and weedy rice from the same or neighbouring farmer's field(s).

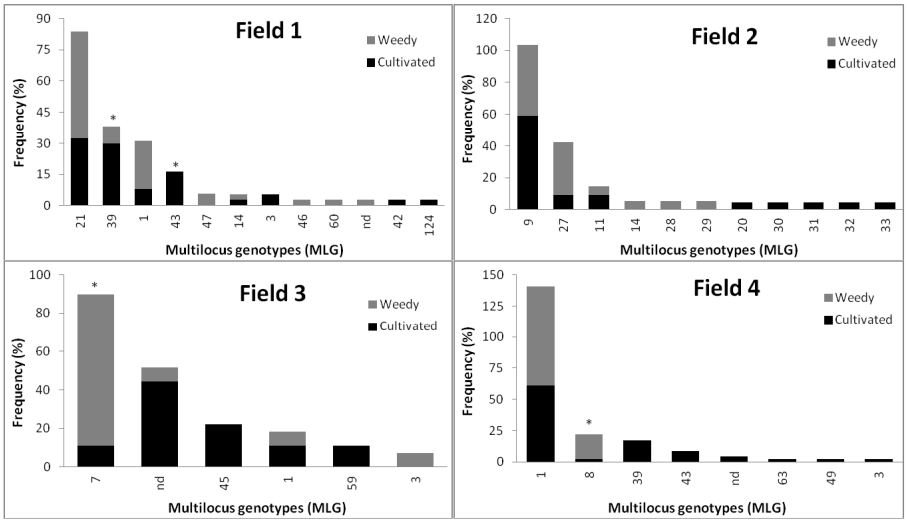


Figure 9.5. Genotypic diversity observed in four rice farmer's fields in France on cultivated and weedy rice. *significant frequency difference between weedy and cultivated rice (Chi² test, P<0.05).

time by comparing the frequency of MLG in France for the periods 1986-1994 (19 strains), 1996-2000 (13 strains) and 2001-2009 (444 strains). Most MLGs prevailing before 1994 were still present (MLG1, 7 and 9; Figure 9.6a**)**. But their frequencies changed significantly. MLGs specific of each period were detected. Contemporary MLG 14 (the most frequently observed around Europe), 39, and 21 were not detected in the previous periods. On the opposite, some others have disappeared long ago or more recently (MLG4, 5, 10, 12, 13, 94, 122 and 123) or are disappearing (MLG2, 6, 8, and 11). Overall, the changing composition of the population seems gradual, some genotypes disappear and some others appear progressively. This evolution was observable on a timescale of 8-10 years. In contrast, the Italian population seems much more stable (Figure 9.6b), even if new MLGs (2001-2009 only) are observed at low frequencies.

Population structure

Since the distribution of MLGs across European countries suggests the existence of migration events, we determined the structure of the fungal population independently of geographic information. Population structure was determined by two complementary methods. First, population structure was inferred using the model-based clustering program STRUCTURE (Pritchard *et al.*, 2000) to define the number of subpopulations, *k*. When *k* was tested from 1 to 10, the posterior probability of the data improved steadily for $k \geq 2$ and reached a plateau for $k = 3$ (data not shown). So, we set the number of subpopulations to three and each MLG was assigned to the subpopulation group according to its probability of membership calculated by STRUCTURE. A graphical display of estimated population structure for $k = 3$ is presented in Figure 9.7a. The results obtained with Principal Component Analysis also indicated the existence of three groups (Figure 9.7b**)**. With a few exceptions,

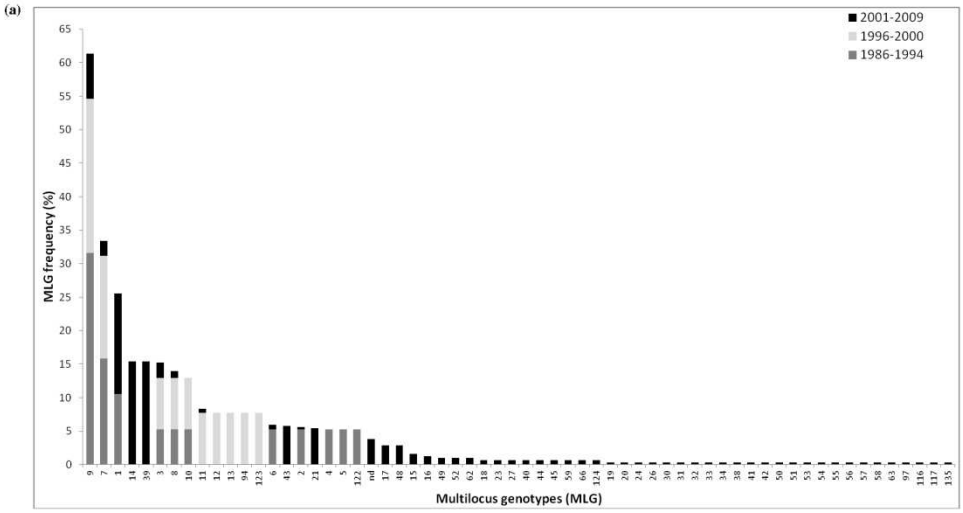


Figure 9.6. Evolution of *Magnaporthe oryzae* polymorphism in France (A) and Italy (B) over the last 25 years.

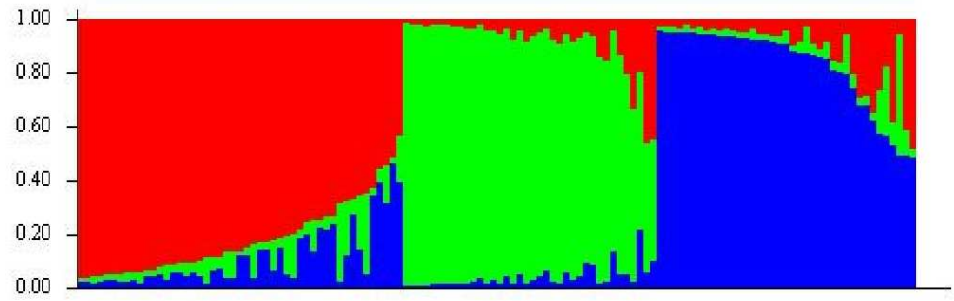


Figure 9.7. Inferred population structure of European *Magnaporthe oryzae* isolates using STRUCTURE model.

this grouping was congruent with that determined by STRUCTURE. Based on these two independent methods, it appears that the European population of *Magnaporthe oryzae* is structured in three main groups. Using STRUCTURE, 305, 441, and 230 isolates were assigned to group 1, 2, and 3 respectively (Table 9.3). Eleven strains (1%) could not be assigned to a defined group. Most of the MLGs collected in France and Italy were spread among the three groups, indicating a shared polymorphism between these countries. Most of the Spanish MLGs were distributed in group 2. Greek MLGs were mainly assigned to group 2 and group 3. The five Turkish multilocus genotypes were classified together in a single group (group 1) as well as the six Hungarian MLGs (group 2). For each country, the observed distribution of isolate number in the three groups was significantly different from the average distribution over all isolates (Table 9.3). For Turkey and Hungary we cannot exclude that this heterogeneity is due to sampling bias: for these countries the sample size is small and probably represents only part of the existing diversity (only one population genotyped for each country). But such sample bias is not expected for France, Italy and Spain, and our results suggest that the distribution of the three groups is not homogeneous over Europe.

A Minimum Spanning Tree was constructed (Figure 9.8) to visualize the genetic relatedness between MLGs. In this tree, 106 MLGs out of 125 were linked together by branches representing a single difference between MLGs. Eight MLGs could not be linked to the tree, including the five Hungarian MLGs. Approximately one fourth of the MLGs of group 1 and of group 3 and most unassigned MLGs (defined with STRUCTURE), were mixed together in the central part of the tree. The remaining MLGs of group 1 were distributed at one edge of the tree. The remaining MLGs of group 3 were distributed in two branches, both being linked to the central part of the tree. All MLGs assigned to group 2 (except two) were located together at the edge opposite to group 1.

	Observed				Expected				
	1	2	3	Total	1	2	3	Chi²	P
FR	214	175	85	474	148.1	214.2	111.7	42.8	0.0000
GR	3	35	30	68	21.3	30.7	16.0	28.5	0.0000
HN		24		24	7.5	10.8	5.7	29.1	0.0000
IT	50	91	63	204	63.8	92.2	48.1	7.6	0.0222
PR	7			7	2.2	3.2	1.6	15.4	0.0005
SP	12	116	52	180	56.3	81.3	42.4	51.8	0.0000
TR	19			19	5.9	8.6	4.5	41.8	0.0000
Total	305	441	230	976				217.0	0.0000

Table 9.3. Distribution of *Magnaporthe oryzae* isolates in the three genetic groups identified in Europe and according to their geographic origin.

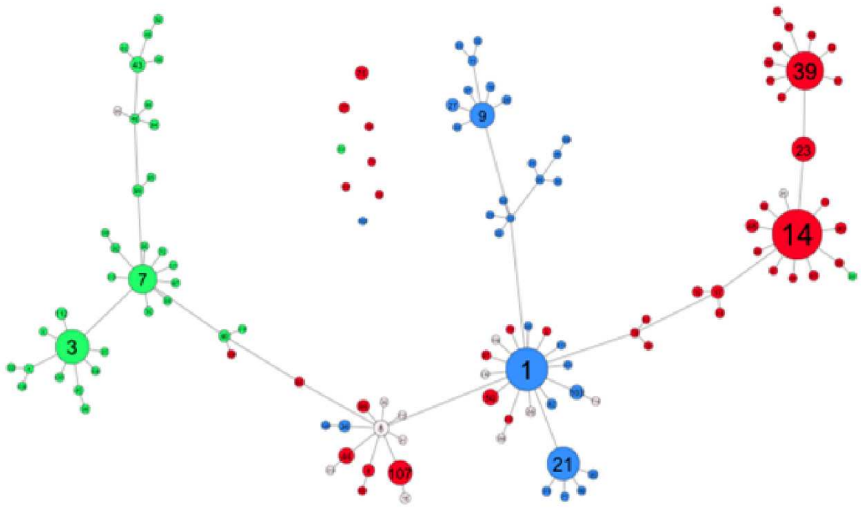


Figure 9.8. Minimum spanning tree of multilocus genotypes (MLGs) representative of *Magnaporthe oryzae* genetic diversity in seven European countries: France, Greece, Hungary, Italy, Portugal, Spain and Turkey. Colors represent the different groups to which MLGs were assigned to with STRUCURE: red, group 1; green, group 2; blue group 3; grey, unassigned. Circle size is proportional to the number of strains in each MLG.

In conclusion, European population of *Magnaporthe oryzae* is structured in three main groups genetically related.

Discussion

Our genetic diversity analysis of 987 European rice blast isolates has shown that, as expected and as already shown in Spain (Lara-Alvarez *et al.*, 2010: 186 rice *M. oryzae* isolates genotyped), increasing considerably the number of strains and the sampling period allowed revealing more genotypes than originally described (Roumen *et al.*, 1997: 41 isolates). The European population is structured in three major genetic groups which were not previously identified. In the three genetic pools were grouped together French, Italian, Spanish and Greek isolates. Previous studies in other temperate rice growing areas such as the USA, Korea, Japan, Iran, Argentina have also shown that population structures were simple and composed of limited lineages (Levy *et al.*, 1993; Don *et al.*, 1999a; Park *et al.*, 2003; Javan-Nikkhah *et al.*, 2004; Consolo *et al.*, 2008). In Europe, six clonal lineages have been identified so far (Jorge 1996; Roumen *et al.*, 1997; Piotti *et al.*, 2005; Lara-Alvarez *et al.*, 2010). In Roumen *et al.* (1997), a clonal lineage was defined as a group of multilocus genotypes which average similarity within a cluster (one lineage) was greater than that between clusters (Rohlf, 1985). Between lineages the average similarity observed ranged from 55 to 65%. Within lineages, similarity was 80% or higher. The difference of structure between previous studies and this study is probably due to the type of markers used and to the difference in sample size. In previous studies, RFLP with repeated dispersed sequence or RAPD markers were used. With such markers, only presence or absence of bands is scored, discriminating only two alleles per locus. In our study, we used microsatellite markers. These markers enable to score several alleles per locus. So, they allow monitoring the occurrence of continuous changes. In our work, we showed the existence of a continuous range of similarity

between MLGs from 20% to 100%. But, in turn, it is also more difficult to define lineages because of this continuity. In addition, the limited number of isolates genotyped in previous studies may have facilitated the definition of more groups (lineages).

Our analyses highlighted that the most frequent MLGs were observed in most of the European countries examined. These data are in agreement with previous results (Jorge, 1996; Roumen *et al.*, 1997) supporting a large distribution of some lineages. Since long distance dispersal by spores is unlikely, this relatively wide distribution of many MLGs between distant rice producing countries could be explained by exchanges of infected seeds between those countries. Indeed, in Europe no phytosanitary control is required for the presence of blast fungus in commercial seeds. Over the last 20 years, most of the new varieties grown in France were also cultivated in Italy before and, because of important seed production capacities, most of the seeds used in France came from Italy. So, the rice seed flow is mostly from Italy to France. If we compare the year of first detection in our sample of the MLGs present in Italy and in France over the last 20 years, 14 MLGs were detected before in Italy *vs.* three in France (data not shown). Intercontinental transfer of plant material by humans, resulting in extensive gene flow, has already been observed for different pathogens (Zhou *et al.*, 2007; Fry, 2008; Robin *et al.*, 2009) and was already suspected for *M. oryzae* (Tharreau *et al.*, 2009). The importance of infected seeds as primary inoculum was already demonstrated for *M. oryzae* (Long *et al.*, 2001).

We compared population structures on weedy and cultivated rice in France to test whether the first one may be a potential source of inoculum to the second. The hypothesis that weedy rice is a primary source of inoculum in Europe cannot be excluded. But, in some cases, some MLGs are specific of either hosts. So, our study revealed that, in some cases, the two populations can sometimes be differentiated. In addition, field observations clearly show that weedy rice is not the unique source of primary inoculum since rice fields devoid of weedy

rice for several years can be severely attacked. In these particular cases, infected seeds are probably the primary source of inoculum, as previously demonstrated (Long *et al.*, 2001).

We also confirmed, with a much larger sample of strains, the existence of some specific MLGs at the country level, suggesting differentiation between European countries. This was the case for France, Spain, Turkey and Hungary. All the Hungarian MLGs were found to be unique. In this particular case, the limited exchanges of rice seeds between Hungary and the other European rice producing countries is sufficient to explain this differentiation. This explanation may also be relevant for Turkey. Between other European countries, the respective role of geographic isolation and/or population selection by specific varieties could explain the specificity observed for some MLGs. When looking at MLGs in the rice production regions within countries, we observed local differentiation such as in Spain between Southern and Northern areas, or in Greece. In Spain, seed exchanges are more frequent between the two Northern areas than between the Northern and Southern areas. In addition, there are more common rice varieties grown between the two Northern areas than between the Northern and Southern areas. Both factors contribute in the same way to the differentiation between Northern and Southern Spanish population of the fungus. Differentiation was observed at even within smaller areas. For example, differentiation between adjacent fields was observed in France. Such differentiation may be due to difference in rice varieties cultivated or in agro-environmental conditions or both. To date, there was very few information on *M. oryzae* diversity at the field scale, especially in farmer's fields. The common assumption was that, most of the time, a unique genotype would invade a field. Our present work shows that although, one or two genotype(s) may usually be predominant in farmer's fields, several additional and genetically related genotypes coexist. So, even at the field level there is some genetic diversity that was previously underestimated. When

reanalyzing the available information (Xia *et al.* 2000), the data support the idea that, although one multilocus genotype may be dominant, there are several MLGs in one field.

In our study we also show evidence for different evolution patterns over time in France and in Italy. In agreement with our data, Piotti *et al.* (2005) reported that the genetic diversity of the blast fungus must have been evolving slowly both within the Italian population and within each lineage. The same authors highlighted that the same lineages were found to be present in Italy for over 30 years with little changes in their virulence. Although, there were changes in varieties in both countries over the last 30 years, the genetic backgrounds probably changed less in Italy than in France. In France, since the 1980s there were two phases of diversification for grain types that leaded to a diversification of donors used in breeding, and, thus to a diversification of genetic backgrounds. These different backgrounds have exerted a selection pressure on the pathogen population that led to the selection of new MLGs. Few studies have been conducted on fungal plant pathogens to investigate changes in population genetic structure over time, with the noticeable exception of *Phytophtora infestans*. For this species, populations were first sampled from several countries in Europe, Asia, South America, and from the USA and Canada prior to the major migrations that began in the latter quarter of the 20th century. These populations were genotypically quite similar and dominated by a single clonal lineage, the US-1 lineage (Goodwin *et al.*, 1994). The global population structure of *P. infestans* began to change in the latter part of the 20th century due to migrations. Three exotic lineages contributed to the severe threat in Northern America during the early 1990s (Fry & Goodwin, 1997). The first two ones disappeared in two to three years. The last one, US-8, appeared in 1992 and spread to most production regions in Northern America in four years. US-8 is still the most widely distributed, dominant, and problematic lineage (Fry, 2008). This means that this lineage was particularly fit, such that it displaced the previous population, and remained as the dominant lineage in the population.

The population structure in three main groups that we detected in Europe is only partially explained by geography. Independent introductions of strains followed by migrations could explain this structure. A strong geographic differentiation of *Cryphonectria parasitica* populations was shown and led to the hypothesis of multiple introductions of this pathogen in Europe (Dutech *et al.*, 2010). Populations in Italy, eastern France, Switzerland, Austria, North-Eastern Spain and Germany would be the result of a first introduction event (Cortesi *et al.*, 1998; Robin *et al.*, 2000; Robin & Heiniger, 2001). In South-Western Europe (Western France, Western Spain and Portugal) other dominant vegetative compatibility (vc) types have been identified (Robin *et al.*, 2000; Aguin *et al.*, 2005; Braganca *et al.*, 2007; Zamora *et al*., 2008). In the Eastern Mediterranean and Balkan countries, more recently invaded by *C. parasitica* (Bosnia Herzegovina, Greece, Macedonia, Slovakia), populations are dominated by a different vc type (Trestic *et al.*, 2001; Sotirovski *et al.*, 2004; Juhasova *et al.*, 2005; Adamcikova *et al*., 2006; Perlerou & Diamandis, 2006). On the contrary to *C. parasitica*, the three groups identified in the European population of *M. oryzae* seem to be genetically related, as shown by the continuous range of similarity observed between MLGs and the structure of the network of haplotypes These results support the hypothesis of a differentiation from a single gene pool for most of the European genotypes. The genotypes found in Hungary may represent an exception to this rule and they may represent an independent introduction in Europe. But further studies will be needed to decipher the introduction and migration events in Europe.

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Annexe 2

Croisements *in vitro* des souches de *Magnaporthe oryzae.*

Le protocole de croisements des souches de *M. oryzae* a été mis au point par Silué et Nottéghem (1992). Ces croisements servent à déterminer le type sexuel (Mat1 ou Mat2) et la capacité à produit les périthèces (femelle-fertile) d'une souche X. La souche est mise en culture en boite de Pétri sur milieu nutritif additionné de farine de riz à partir d'un stock papier, et placée à 25°C avec alternance de lumière de 12h. Au même moment, des souches de référence sont aussi mises en culture. Ce sont des souches dont le type sexuel est connu et qui sont femelle-fertiles, ainsi l'on sait qu'elles peuvent produire des périthèces au contact d'une souche de type sexuel opposé. Au bout d'une semaine, deux cubes de gélose sont prélevés de la souche X à l'aide d'un scalpel : un cube est placé sur une nouvelle boite de Pétri avec des cubes de gélose provenant de deux souches de référence Mat1 et un cube est placé sur une nouvelle boite de Pétri avec des cubes de gélose provenant de deux souches de référence Mat2 (Figure 10.1). Sur chaque boite, on peut tester deux souches en même temps (X et Y). Les boites de Pétri et celle-ci est placée à 20°C pendant deux à trois semaines en lumière continue. Au bout de ce délai, les souches ont colonisé la boite et la formation des périthèces sur les lignes de confrontations entre les souches testées et les deux souches de référence peut être observée. Plusieurs cas de figure peuvent se présenter (Figure 10.2). Si la souche X est Mat1, alors on observera des périthèces dans la boite contenant les souches de référence Mat2, et si elle est Mat2 les périthèces seront observés dans la boite contenant les souches de référence Mat1. Si en plus la souche X est femelle-fertile, on observera deux rangées de périthèces correspondant à ceux produit par la souche X et ceux produits par les souches de référence. Si elle est femelle-stérile, alors on n'observera qu'une seule rangée de périthèces : ceux produits par les souches de référence. Certaines souches sont incapables d'induire la production de périthèces par les souches de références. Dans ce cas aucun périthèce n'est observé dans aucune condition.

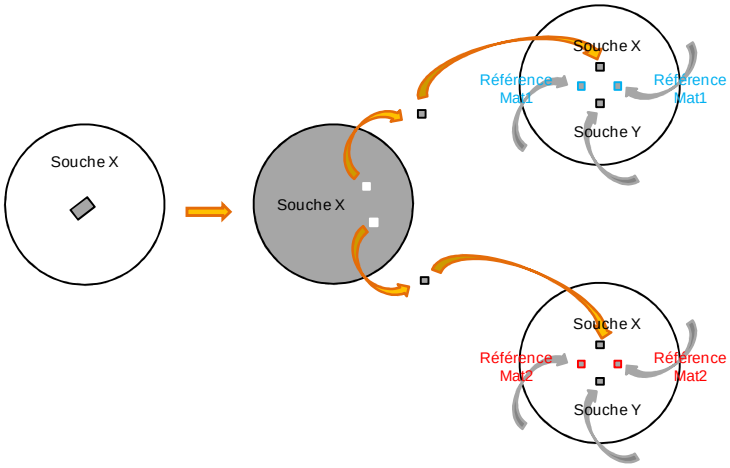


Figure 10.1. Protocole de croisement de *Magnaporthe oryzae*.

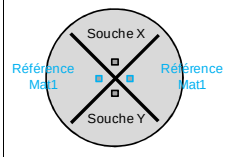
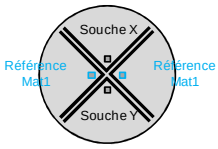
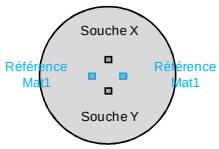
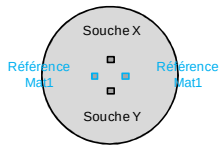
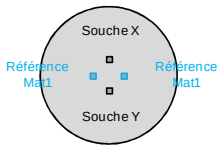
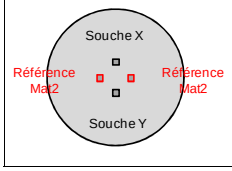
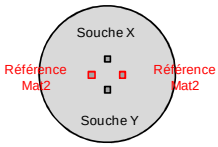
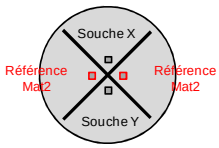
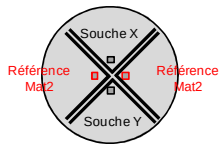
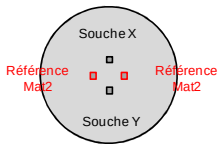
X et Y sont Mat2		X et Y sont Mat1		Le type sexuel est inconnu
X et Y sont femelle-stériles	X et Y sont femelle-fertiles	X et Y sont femelle-stériles	X et Y sont femelle-fertiles	X et Y sont mâle-stériles et femelle-stériles
				
				

Figure 10.2. Résultats possibles de croisements de *Magnaporthe oryzae*.

Annexe 3
Evolution expérimentale *in vitro* des souches de *Magnaporthe oryzae.*

Le protocole d'évolution expérimentale que nous avons mis en place a eu pour but d'étudier la production de périthèces par des souches femelle-fertiles quand elles sont forcées à se reproduire de manière exclusivement clonale pendant 10 à 20 générations. Le protocole a été le même pour chaque souche.

Une souche a été cultivée pendant une semaine sur milieu additionné de farine de riz pendant une semaine à 25°C avec une alternance de lumière de 12h. La boite a ensuite été tapée sur une deuxième boite de Pétri afin de faire tomber les conidies (Figure 11.1). Un stock de la souche a été réalisé à partir de la première boite sur papier filtre et est figée à -20°C. La deuxième boite a été soumise aux mêmes conditions que la première pendant une semaine puis a été tapée à son tour sur une troisième boite. Un deuxième stock a été réalisé à partir de la deuxième boite. Ainsi, la manipulation a été réalisée 10 à 20 fois. A la fin de l'expérience les souches stockées ont été croisées avec des souches de référence (voir Annexe 2 pour le protocole de croisement) et la production de périthèces a été estimée sur trois carrés de $9mm^2$ de surface avec chaque souche de référence (Figure 11.2). La diminution de la production de périthèces est visible à l'œil nu (Figure 11.3).

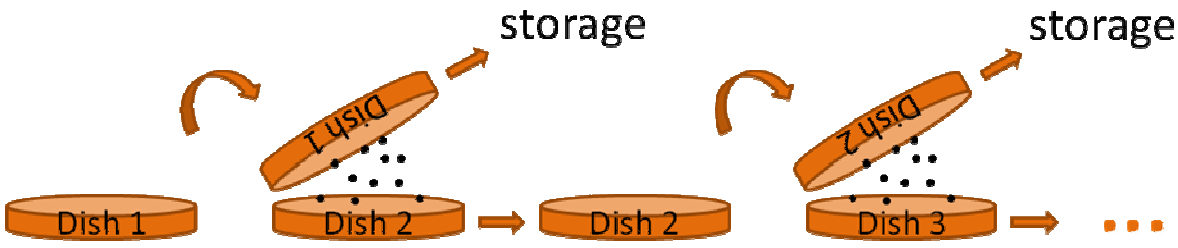


Figure 11.1. Protocole d'évolution expérimentale.

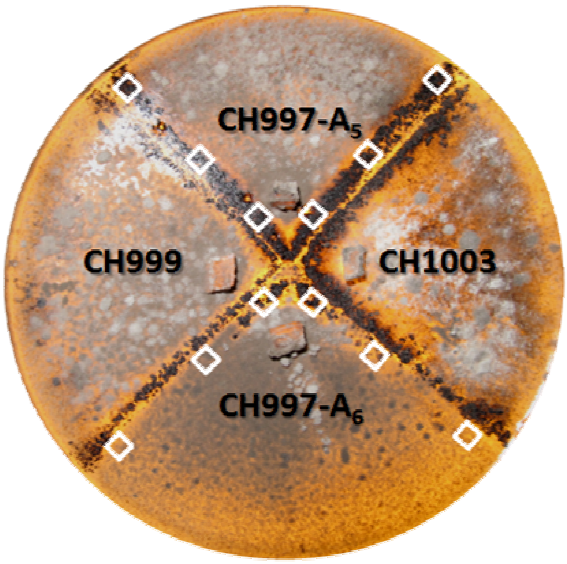


Figure 11.2. Estimation du nombre de périthèces produits par la souche CH997 au cours de son évolution expérimentale.

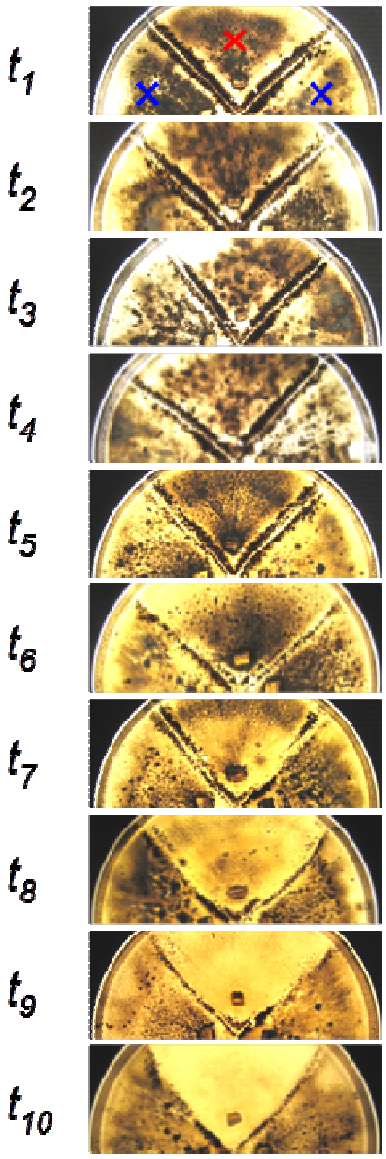


Figure 11.3. Diminution de la fertilité-femelle (et de la fertilité mâle) au cours de l'évolution expérimentale *in vitro* de la souche CH997 de *M. oryzae* pendant 10 générations clonales. Une génération clonale correspond ici à la croissance de la souche entre deux transferts de conidies d'une boite de Pétri à une autre, c'est-à-dire 7 jours. A chaque génération clonale la souche a été confrontée, sur une même boite de Pétri, à deux souches de références de type sexuel opposé à CH997, c'est-à-dire Mat1, et femelle-fertiles. Les zones symbolisées par les croix bleues correspondent aux zones de croissance des souches de référence (CH999 à gauche et CH1003 à droite). La zone symbolisée par la croix rouge correspond à la zone de croissance de CH997. Au début de l'expérience, deux rangées de périthèces sont produites entre CH997 et chacune des souches de référence : un rangée est produite par CH997 puisqu'elle est femelle-fertile et une rangée est produite par chaque souche de référence. La fertilité femelle diminue rapidement jusqu'à devenir nulle au temps t_{10} puisque l'on n'observe plus qu'une seule rangée de périthèces. La fertilité mâle diminue aussi mais n'est pas supprimée.